Alteration in gene expression of branched-chain keto acid dehydrogenase kinase but not in gene expression of its substrate in the liver of clofibrate-treated rats

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We previously showed that the oxidation of branched-chain amino acids is increased in rats treated with clofibrate [Paul and Adibi (1980) J. Clin. Invest. **65**, 1285–1293]. Two subsequent studies have reported contradictory results regarding the effect of clofibrate treatment on gene expression of branched-chain keto acid dehydrogenase (BCKDH) in rat liver. Furthermore, there has been no previous study of the effect of clofibrate treatment on gene expression of BCKDH kinase, which regulates the activity of BCKDH by phosphorylation. The purpose of the present study was to investigate the above issues. Clofibrate treatment for 2 weeks resulted in (a) a 3-fold increase in the flux through BCKDH in mitochondria isolated from rat liver, and (b) a modest but significant increase in the activity of BCKDH. However, clofibrate treatment had no significant effect on the mass of $E_1\alpha$, $E_1\beta$, and E_2 subunits of BCKDH or the abundance of mRNAs encoding these subunits. On the other hand, clofibrate treatment significantly reduced the activity, the protein mass and the mRNA levels of BCKDH kinase in the liver. In contrast to the results obtained in liver, clofibrate treatment had no significant effect on any of these parameters of BCKDH kinase in the skeletal muscle. In conclusion, our results show that clofibrate treatment increases the activity of BCKDH in the liver and the mechanism of this effect is the inhibition of gene expression of the BCKDH kinase.

INTRODUCTION

Branched-chain keto acid dehydrogenase (BCKDH) is the key enzyme regulating the oxidation of branched-chain amino acids, leucine, isoleucine and valine [1,2]. BCKDH is composed of three catalytic proteins, namely a branched-chain keto acid decarboxylase (E_1), a dihydrolipoyl transacylase (E_2) and a dihydrolipoyl dehydrogenase (E_3) [3–7]. The E_1 component is further composed of α ($E_1\alpha$) and β ($E_1\beta$) subunits [8]. The E_1 and E_2 components are specific for BCKDH, whereas E_3 is common to BCKDH, pyruvate and α-oxoglutarate dehydrogenase complexes [9]. It is generally believed that the activity of this enzyme is altered by a change in phosphorylation-dephosphorylation of the enzyme protein and not by a change in gene expression [10]. Recently this view became untenable when several studies demonstrated changes in BCKDH activity caused by altered gene expression [11–14]. Among the agents reported to affect gene expression of BCKDH was the hypolipidaemic drug, clofibrate. Ono et al. [14] reported that clofibrate treatment increased the gene expression of this enzyme in rat liver. However, the results of the study of Ono et al. could not be confirmed by Zhao et al. [15].

In view of the importance of the above issue, an additional study appeared to be necessary to resolve the conflicting findings. Therefore, in the present study, we have re-investigated the effect of clofibrate treatment on BCKDH activity, the protein mass of BCKDH subunits, and the relative abundance of mRNAs encoding the $E_1\alpha$, $E_1\beta$ and E_2 subunits of BCKDH.

Having found that the clofibrate treatment increases the activity of BCKDH in the liver, we also investigated an alteration in the activity of BCKDH kinase as a possible mechanism. The effect of clofibrate treatment on the protein mass and gene expression of the kinase, which catalyses the phosphorylation and inactivation of BCKDH, has not been previously studied.

EXPERIMENTAL

Materials

NAD^+, CoASH, thiamine pyrophosphate, *N*-2-*p*-tosyl-L-lysyl-chloromethane, leupeptin, trypsin inhibitor, pepstatin, aprotinin, 4-methyl-2-oxopentanoic acid, 2-oxopentanoic acid and dihydrolipoyl dehydrogenase were obtained from Sigma Chemical Company. Catalase and L-amino acid oxidase were obtained from Boehringer-Mannheim. Dichloroacetic acid was obtained from Eastman Kodak. GeneScreen membranes and [α-^{32}P]dCTP were from New England Nuclear. Kit for radiolabelling DNA was obtained from Pharmacia. Films for autoradiography and chemiluminescence were obtained from Kodak and Amersham, respectively. L-[1-^{14}C]Leucine (54 mCi/mmol) and the enhanced chemiluminescence (ECL) Western blotting kit were purchased from Amersham Corp. Clofibrate (Atromid S) was obtained from Ayerst Laboratories, New York. 4-Methyl-[1-^{14}C]2-oxopentanoic acid was prepared in this laboratory from L-[1-^{14}C]leucine by incubating with L-amino acid oxidase as described by Rudiger et al. [16]. All other reagents were of analytical grade and were obtained from Sigma. BCKDH was purified from rat liver as described previously [15]. Polyclonal antibodies against purified rat liver BCKDH were raised in rabbits by Pel-Freez Biologicals (Rogers, AR). BCKDH kinase was purified from rat liver as outlined by Shimomura et al. [17]. Polyclonal antibodies

Abbreviations used: BCKDH, branched-chain keto acid dehydrogenase; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEG, poly(ethylene glycol).

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against the purified kinase were raised in rabbits by Pel-Freez Biologicals. A broad-specificity phosphoprotein phosphatase was purified from rat liver as described by Brandt et al. [18] and Gillim et al. [19]. Cloned cDNAs encoding the $E_1\alpha$, $E_1\beta$ and E_2 subunits of rat BCKDH and BCKDH kinase were kindly provided by Dr. Robert Harris, Indiana University School of Medicine. Additional cDNA clones used encoded rat malic enzyme [20] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [21].

Animals and their treatment

Male Sprague–Dawley rats weighing approx. 200 g were used in this study. Rats were housed in individual cages in air-conditioned quarters with a controlled 12-h light/dark cycle and received powdered Purina Laboratory Chow and drinking water *ad libitum*. Rats were fed for 2 weeks on a diet containing clofibrate (0.25 % of diet).

Isolation of liver mitochondria and measurement of flux through BCKDH

Mitochondria were isolated as outlined by Johnson and Lardy [22]. Flux through BCKDH was determined by measuring the release of $^{14}CO_2$ from 4-methyl-[1-^{14}C]2-oxopentanoic acid incubated with mitochondria as reported previously [23].

Extraction and assay of BCKDH activity

BCKDH from liver was extracted by a poly(ethylene glycol) (PEG) precipitation method as outlined by Goodwin et al. [24]. The activity of BCKDH was determined spectrophotometrically by measuring the reduction of NAD^+ as described previously [11]. Complete assay mixture contained, in a final volume of 2 ml, 30 mM potassium phosphate buffer, pH 7.4, 3 mM NAD^+, 0.4 mM CoASH, 0.4 mM thiamine pyrophosphate, 2 mM dithiothreitol (DTT), 5 mM Mg^{2+}, 10 units of pig heart dihydrolipoyl dehydrogenase, 0.1 % (v/v) Triton X-100, 0.5 mM 2-oxopentanoic acid and appropriate amounts of BCKDH (0.5–1.0 mg of protein). Assays were performed at 30 °C, and the enzyme activity is expressed as nmol of NADH formed per g wet weight of liver.

BCKDH extraction and Western blot analysis

BCKDH was quantitatively precipitated by the PEG method described above. Identical amounts of BCKDH precipitate from livers of control and clofibrate-fed rats were suspended in SDS buffer [4 % (w/v) SDS, 0.125 M Tris/HCl, pH 6.8, 20 % (v/v) glycerol and 0.125 % (w/v) DTT] [15] and boiled for 90 s. Samples were subjected to SDS/10 % PAGE in the system of Laemmli [25] and transferred to nitrocellulose membranes by electroblotting. The nitrocellulose membranes were blocked with non-fat milk in PBS-T buffer [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.02 % NaN_3, 0.1 % (v/v) Tween-20 and 0.5 % (w/v) non-fat milk, pH 7.2] for 1 h at room temperature. Next, the nitrocellulose membrane was incubated with polyclonal antisera (1:2000) raised against purified rat BCKDH complex in PBS-T buffer for 1 h at room temperature. After incubating with the first antibody, the nitrocellulose membrane was washed in PBS-T buffer for two 10-min periods and incubated with a second antibody [peroxidase-conjugated goat anti-(rabbit IgG) antibodies; 1:2000]. Subunits of BCKDH complex were detected with the ECL Western blotting system of Amersham following the manufacturer's instructions. The intensity of bands was quantified by densitometry using a Bio-Rad Model 620 densitometer. To determine the linearity of the Western blot assay, in preliminary studies, total protein in the range of 50–250 μg was subjected to electrophoresis and Western blot analysis. The linearity of the Western blot assay was determined by densitometric scanning of the resulting film. The results of the assay were linear from 50 to 250 μg of total protein for the $E_1\alpha$ subunit and from 50 to 150 μg of total protein for $E_1\beta$ and E_2 subunits of BCKDH (results not shown). The correlation coefficients between the amount of protein and ECL image intensity were 0.95, 0.91 and 0.99 for $E_1\alpha$, $E_1\beta$ and E_2 subunits of BCKDH respectively (all $P < 0.01$). Based on these results, 100 μg of total protein was used for all subsequent Western blot analyses.

RNA extraction and Northern blot analysis

Total RNA from liver or skeletal muscle (gastrocnemius) was extracted by a guanidine thiocyanate method [26]. RNA (10 μg for BCKDH complex and 25 μg for BCKDH kinase) was fractionated on a 0.9 % (w/v) agarose gel containing formaldehyde, blotted to GeneScreen membranes, and hybridized as described previously [27]. ^{32}P-labelled cDNA probes were made by the random primer technique [28]. Blots were subjected to autoradiography in the presence of intensifying screens using preflashed X-ray film [29]. The intensity of bands was quantified by densitometry using a Bio-Rad Model 620 densitometer. mRNA levels for each sample were normalized to the abundance of GAPDH mRNA, which served as an internal control for minor variations in sample loading.

Isolation of hepatic BCKDH-kinase complex

A BCKDH-kinase complex retaining high kinase activity was isolated by slightly modified procedures of Shimomura et al. [17] and Popov et al. [30]. Liver was frozen and pulverized under liquid nitrogen. The fine powder was suspended (1:9, w/v) in Buffer A [30 mM Hepes, 1 mM EDTA, 0.15 M KCl, 0.5 % (w/v) Triton X-100, 2 % (v/v) rat serum, 0.1 mM *N*-tosyl-L-lysylchloromethane, 10 μg/ml trypsin inhibitor, 1 μM leupeptin, 3 mM DTT and 0.1 mM thiamine pyrophosphate, pH 7.4], frozen and thawed. The thawed suspension was homogenized with a Polytron homogenizer for 30 s in ice at a low setting. The suspension was centrifuged at 15000 ***g*** for 5 min, the supernatant fraction saved and its volume measured. To this supernatant fraction, 27 % (w/v) PEG was added to give a final PEG concentration of 1 %. After stirring on ice for 20 min, the suspension was centrifuged at 15000 ***g*** for 10 min, the supernatant fraction saved and its volume measured. To this supernatant fraction, an additional amount of 27 % PEG was added to give a final PEG concentration of 5 %. After stirring on ice for 20 min, the suspension was centrifuged at 15000 ***g*** for 10 min and the pellet containing the PEG precipitate was saved. This pellet was resuspended (1:15, w/v of initial liver weight) in Buffer B [30 mM Hepes, 0.1 mM EDTA, 0.1 M KCl, 10 mM $MgSO_4$, 0.1 % (w/v) Triton X-100, 10 μg/ml trypsin inhibitor, 1 μM leupeptin and 5 mM DTT, pH 7.4] and homogenized with a Dounce homogenizer. The suspension was incubated at 37 °C for 1 h. After 1 h, the suspension was chilled on ice and 27 % (w/v) PEG added to give a final PEG concentration of 4 %. After stirring on ice for 20 min, the suspension was centrifuged at 15000 ***g*** for 10 min and the pellet containing the PEG precipitate was saved. The pellet was resuspended (1:4, w/v of initial liver weight) in Buffer C (30 mM Hepes, 5 mM DTT, 0.1 mM EDTA, 0.05 % Triton X-100, 1 μM leupeptin and 10 μg/ml trypsin

inhibitor, pH 7.4) using a Dounce homogenizer. This suspension served as the source of BCKDH–kinase complex.

BCKDH kinase assay

BCKDH kinase activity was assayed by measuring the rate of loss of BCKDH activity in the presence of ATP as described by Shimomura et al. [17] and Popov et al. [30]. The complete reaction mixture contained, in a final volume of 0.20 ml, 30 mM Hepes, 1.5 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 0.05% (w/v) Triton X-100, 0.1 μM leupeptin, 10 μg/ml trypsin inhibitor, 0.5 mM ATP, pH 7.35, and 0.10 mg of BCKDH–kinase complex. Reactions were incubated at 30 °C for 10 min. At various time intervals (0–10 min), aliquots (20 μl) were removed and transferred into BCKDH assay mixture and dehydrogenase activity was measured spectrophotometrically as described above.

Hepatic BCKDH–kinase Western blot analysis

BCKDH–kinase complex was extracted from the liver and precipitated with PEG as described above for BCKDH. Identical amounts of BCKDH–kinase precipitate from livers of control and clofibrate-fed rats were suspended in the SDS buffer described above for BCKDH Western blot analysis. Samples were subjected to SDS/10%-PAGE [25] and transferred to nitrocellulose membranes by electroblotting. The nitrocellulose membrane was incubated with polyclonal antisera (1:500) raised against purified rat BCKDH kinase in PBS-T buffer for 1 h at room temperature. After incubating with the first antibody, the nitrocellulose membrane was washed in PBS-T buffer for two 10-min periods and incubated with a second antibody [peroxidase-conjugated goat anti-(rabbit IgG) antibodies, 1:1000]. BCKDH kinase was detected with the ECL Western blotting system of Amersham following the manufacturer's instructions. The intensity of bands was quantified by densitometry using a Bio-Rad Model 620 densitometer. To determine the linearity of the Western blot assay, in preliminary studies, total protein in the range of 75–250 μg was subjected to electrophoresis and Western blot analysis. The linearity of the Western blot assay was determined by densitometric scanning of the resulting film. The results of the assay were linear from 75 to 225 μg of total protein. The correlation coefficient between the amount of protein and ECL image intensity was 0.99 ($P < 0.01$). Based on these results, 100 μg of total protein was routinely used for all subsequent Western blot analysis.

BCKDH–kinase extraction from skeletal muscle and Western blot analysis

BCKDH–kinase complex was isolated from the gastrocnemius muscle by a PEG precipitation method as described previously [31]. Western blot analysis was performed as described above for the kinase from the liver.

Analytical methods

Total peroxisomal β-oxidation activity was measured in the 600 g supernatant fraction of liver homogenates using the palmitoyl-CoA-dependent, potassium cyanide-insensitive, NAD reduction as described by Lazarow [32]. Protein concentration was determined by the Lowry method [33] using BSA as the standard.

Statistics

All data are presented as mean $\pm$ S.E.M. Student's t test [34] was used for statistical analysis of data.

RESULTS

Clofibrate-treated rat model

Clofibrate treatment is well known to induce hepatomegaly [35] and hepatic proliferation of peroxisomes [36]. Therefore, to validate the effectiveness of clofibrate treatment under the present experimental conditions, we initially measured liver weights and total peroxisomal fatty acid β-oxidation activity, a marker of peroxisomal proliferation [37,38], in six control and six clofibrate-treated rats. Clofibrate treatment, significantly ($P < 0.01$) increased liver weights (4.22 ± 0.08 versus 6.20 ± 0.15% of body weight) and total peroxisomal β-oxidation activity (7.20 ± 0.36 versus 52.61 ± 7.45 nmol/min per mg of protein) thus establishing the validity of our model.

Flux through BCKDH in liver mitochondria

A previous study [14], using a liver mitochondrial preparation, reported a 3-fold increase in the flux through BCKDH in rats treated with clofibrate. To investigate the reproducibility of this observation, we performed a similar study, using liver mitochondria from six control and six clofibrate-treated rats. We found that, indeed, clofibrate treatment significantly ($P < 0.01$) increased the flux through BCKDH by approx. 3-fold (29.6 ± 2.5 versus 83.0 ± 3.6 nmol/mg of protein per 30 min).

Activity of BCKDH

To determine whether an increase in the basal activity of BCKDH was responsible for the increased flux, we determined the basal activity of BCKDH in livers of five control and five clofibrate-treated rats. Indeed, clofibrate treatment significantly ($P < 0.05$) increased the basal activity of BCKDH (1634 ± 28 versus 1847 ± 61 nmol/min per g of liver).

Protein mass of BCKDH subunits

An increase in the basal BCKDH activity may be due to an increase in the amount of enzyme. To investigate this possibility, we determined the effect of clofibrate treatment on BCKDH subunit mass using quantitative Western blot analysis. As shown in Figure 1(A), the amounts of $E_1\alpha$, $E_1\beta$, and E_2 subunits of BCKDH detected by immunoblot analysis were similar between control and clofibrate-treated rat livers. To quantify changes in the relative abundance of BCKDH subunits in response to clofibrate, the immunoblots were subjected to densitometric analysis. There were no significant differences in amounts of $E_1\alpha$, $E_1\beta$ and E_2 subunits of BCKDH between control and clofibrate-treated rats (Figure 1B). These results support those of Zhao et al. [15], but contradict those of Ono et al. [14].

Gene expression of BCKDH subunits

To determine whether, as reported by Ono et al. [14], clofibrate treatment increases the gene expression of BCKDH, we measured

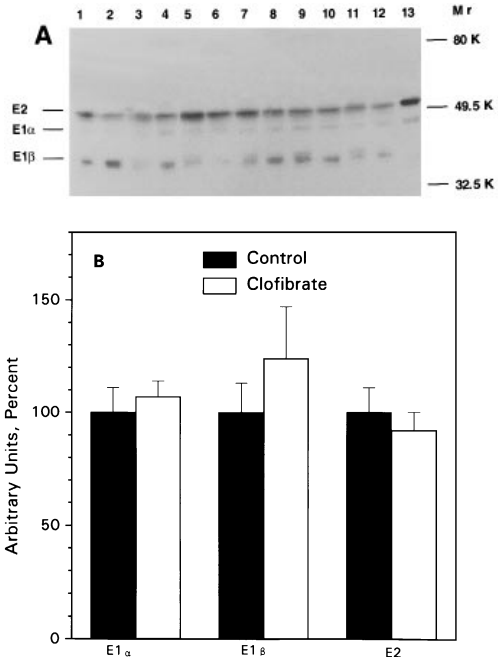

Figure 1 Western blot analysis of $E_1\alpha$, $E_1\beta$ and E_2 subunits of BCKDH isolated from livers of control and clofibrate-treated rats

BCKDH complex was extracted from livers of control and clofibrate-treated rats and subjected to SDS/PAGE. Resolved proteins were transferred to nitrocellulose membranes and subjected to immunoblot analysis using polyclonal antibodies against rat BCKDH. Subunits of BCKDH were detected by ECL using peroxidase-conjugated goat anti-(rabbit IgG) antibodies. (**A**) Lanes 1, 3, 5, 7, 9 and 11 represent subunits of BCKDH from livers of six individual control rats; lanes 2, 4, 6, 8, 10 and 12 represent subunits of BCKDH from livers of six individual clofibrate-treated rats; lane 13 represents purified BCKDH. (**B**) Quantitative densitometric analysis of Western blot for $E_1\alpha$, $E_1\beta$ and E_2 subunits of BCKDH. Values are presented as mean ± S.E.M. of six rats and are expressed as percentages of controls.

Table 1 Effect of clofibrate treatment on Northern blot analysis of mRNA encoding $E_1\alpha$, $E_1\beta$ and E_2 subunits of BCKDH and malic enzyme in rat liver

Results are expressed as mean ± S.E.M. of 5–6 rats.

	mRNA levels relative to controls			
Treatment	$E_1\alpha$	$E_1\beta$	E_2	Malic enzyme
Control	1.00 ± 0.12	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.21
Clofibrate	1.15 ± 0.08	0.95 ± 0.22	0.96 ± 0.13	4.92 ± 0.59*

* Significantly different from control at $P < 0.01$.

the levels of mRNAs encoding BCKDH subunits by Northern blot analysis. Clofibrate treatment did not affect the mRNA levels for any of the BCKDH subunits (Table 1). As a positive control for the clofibrate effect, we measured the levels of malic enzyme mRNA. In contrast to the mRNA levels for the BCKDH subunits, clofibrate treatment significantly increased the mRNA levels for malic enzyme (Table 1). These results support the finding of Zhao et al. [15], but contradict that of Ono et al. [14].

Ono et al. [14] had used male Wistar rats for their studies whereas in the present study male Sprague–Dawley rats were used. To determine whether the strain of rats used accounted for the differences in results between the two studies, we investigated

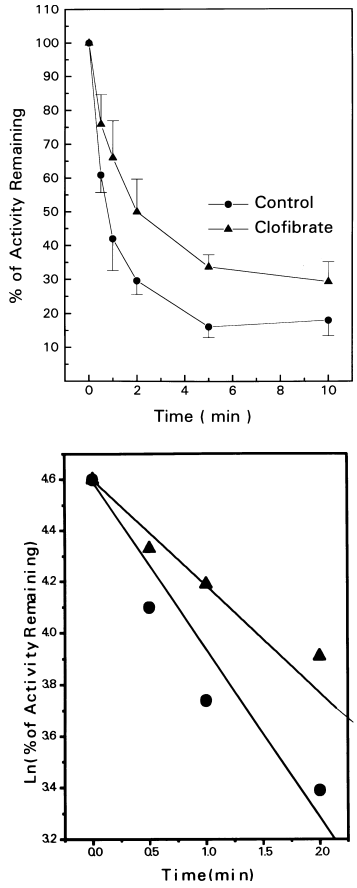

Figure 2 Effect of clofibrate treatment on BCKDH kinase activity in the liver

Upper panel: BCKDH kinase was assayed by measuring the ATP-dependent inactivation of BCKDH. Results are mean ± S.E.M. of five rats. Lower panel: The initial (0–2 min) rate of the kinase reaction is plotted on a logarithmic scale.

the effect of clofibrate also using Wistar rats. Similar results were obtained with Wistar rats as with Sprague–Dawley rats (results not shown).

Activity and protein mass of BCKDH kinase in the liver

To investigate whether the increase in the BCKDH activity reflected a decrease in the phosphorylation of this enzyme by clofibrate treatment, we determined the activity of BCKDH kinase. As shown in Figure 2, clofibrate treatment, indeed, decreased the activity of BCKDH kinase in the liver. To determine whether the decreased kinase activity produced by clofibrate treatment reflected a change in the kinase mass, we performed quantitative Western blot analysis. The amount of BCKDH kinase detected by immunoblot analysis was lower in livers of clofibrate-treated rats than control rats (Figure 3A). To quantify changes in the kinase mass in response to clofibrate, the immunoblots were subjected to densitometric analysis. There was a 66% decrease in the mass of BCKDH kinase in livers of clofibrate-treated rats (Figure 3B).

Gene expression of BCKDH kinase in the liver

To determine whether a decrease in the BCKDH kinase mass in response to clofibrate treatment (Figure 3) reflected regulation at

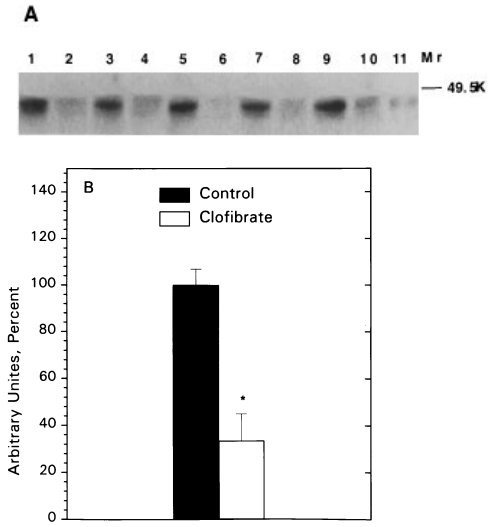

Figure 3 Western blot analysis of BCKDH kinase from livers of control and clofibrate-treated rats

BCKDH–kinase complex was extracted from livers of control and clofibrate-treated rats and subjected to SDS/PAGE. Resolved proteins were transferred to nitrocellulose membranes and subjected to immunoblot analysis using polyclonal antibodies against purified rat liver BCKDH kinase. BCKDH kinase was detected by ECL using peroxidase-conjugated goat anti-(rabbit IgG) antibodies. (**A**) Lanes 1, 3, 5, 7 and 9 represent BCKDH kinase from livers of five individual control rats; lanes 2, 4, 6, 8 and 10 represent BCKDH kinase from livers of five individual clofibrate-treated rats; lane 11 represents purified BCKDH kinase. (**B**) Quantitative densitometric analysis of Western blot. Values are presented as mean ± S.E.M. of five rats and are expressed as percentages of controls. The reduction in the BCKDH kinase mass in livers of clofibrate-treated rats is statistically significant ($*P < 0.01$).

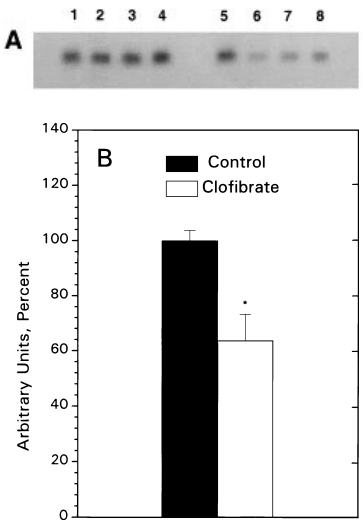

Figure 4 Northern blot analysis of mRNA for BCKDH kinase from livers of control and clofibrate-fed rats

(**A**) Total RNA was extracted and subjected to Northern blot analysis using ^{32}P-labelled cDNA for rat BCKDH kinase. Lanes 1–4 represent mRNA from livers of four control rats; lanes 5–8 represent mRNA from livers of four clofibrate-fed rats. (**B**) Densitometric analysis of Northern blot. Values are mean ± S.E.M. of livers from four animals and are expressed as percentages of controls. The reduction in the levels of kinase mRNA in livers of clofibrate-treated rats is statistically significant ($*P < 0.01$).

the pretranslational step, we measured the abundance of mRNA encoding this kinase. Northern blot analysis demonstrated that the levels of mRNA for BCKDH kinase were decreased in livers of clofibrate-fed rats (Figure 4A). To quantify changes in the levels of kinase mRNA in response to clofibrate, the Northern blots were subjected to densitometric analysis. There was a 40 % decrease in the levels of kinase mRNA in livers of clofibrate-fed rats (Figure 4B).

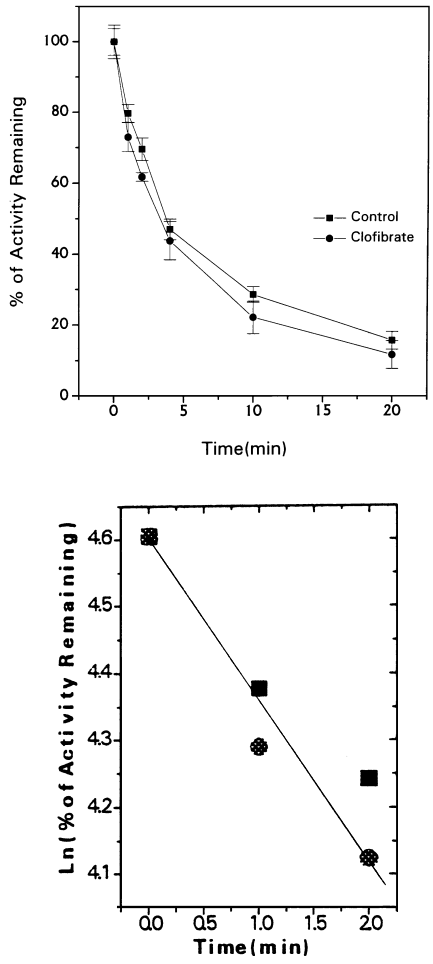

Figure 5 Effect of clofibrate treatment on BCKDH kinase activity in skeletal muscle

Upper panel: BCKDH kinase was assayed by measuring the ATP-dependent inactivation of BCKDH. Results are mean ± S.E.M. of five rats. Lower panel: The initial (0–2 min) rate of kinase reaction is plotted on a logarithmic scale.

Tissue-specificity of the clofibrate effect on BCKDH kinase

The effect of clofibrate treatment appeared to be specific to the liver since it did not affect either the activity (Figure 5), the protein mass (Figure 6), or the mRNA levels of BCKDH kinase (Figure 7) in skeletal muscle.

DISCUSSION

We previously showed that the oxidation of branched-chain amino acids is increased in rats treated with clofibrate [39]. A possible mechanism could be the induction of BCKDH which is the key enzyme regulating this oxidation. The present study shows that clofibrate treatment does not result in the induction of BCKDH in the rat liver, as shown by lack of any effect either on protein mass (Figure 1) or on gene expression (Table 1) of this

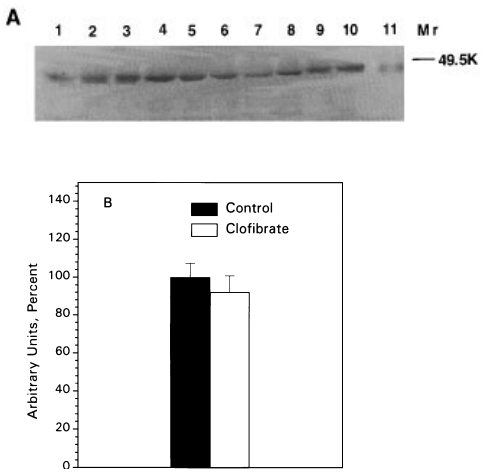

Figure 6 Western blot analysis of BCKDH kinase from skeletal muscle of control and clofibrate-treated rats

BCKDH–kinase complex was isolated from skeletal muscles of control and clofibrate-treated rats and subjected to SDS/PAGE. Resolved proteins were transferred to nitrocellulose membranes and subjected to immunoblot analysis using polyclonal antibodies against purified rat liver BCKDH kinase. BCKDH kinase was detected by ECL using peroxidase-conjugated goat anti-(rabbit IgG) antibodies. (**A**) Lanes 1–5 represent BCKDH kinase from muscles of five individual control rats; lanes 6–10 represent BCKDH kinase from muscles of five individual clofibrate-treated rats; lane 11 represents purified BCKDH kinase. (**B**) Quantitative densitometric analysis of Western blot. Values are presented as mean ± S.E.M. of five rats and are expressed as percentages of controls.

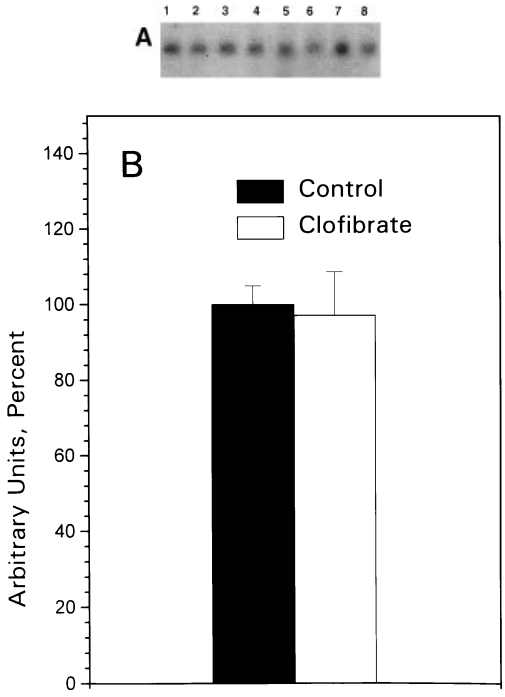

Figure 7 Northern blot analysis of mRNA for BCKDH kinase from skeletal muscle of control and clofibrate-treated rats

(**A**) Total RNA was extracted and subjected to Northern blot analysis using ^{32}P-labelled cDNA for rat BCKDH kinase. Lanes 1–4 represent mRNA from skeletal muscle of four control rats; lanes 5–8 represent mRNA from skeletal muscle of four clofibrate-fed rats. (**B**) Densitometric analysis of Northern blot. Values are mean ± S.E.M. of skeletal muscle from four animals and are expressed as percentages of controls.

enzyme. However, the present results confirm the findings of Ono et al. [14] and Zhao et al. [15] that clofibrate treatment increases the flux through BCKDH and also increases the activity of BCKDH in the rat liver, respectively. However, we find that these increases are not proportional; a 3-fold increase in the flux versus a modest increase in the enzyme activity. We speculate that this may be related to the endogenous levels of CoA, a cofactor for BCKDH. Clofibrate treatment has been shown to increase the hepatic levels of free CoA by nearly 5-fold [40]. Therefore, flux through BCKDH with liver mitochondria from clofibrate-treated rats is measured at a higher concentration of CoA relative to the mitochondria from livers of control rats. In contrast to flux, the activity of BCKDH isolated from livers of both control and clofibrate-treated rats is measured at the same and optimum concentration of exogenously added CoA. The above speculation also implies that the level of free CoA may be limiting for the maximum flux rate through BCKDH.

The present study provides novel information on the effect of clofibrate treatment on the activity of BCKDH kinase in the liver. Although Zhao et al. [15] did not find any alteration in the activity of this enzyme in the liver of clofibrate-treated rats, we found a significant decrease (Figure 2). Our observation was further supported by a significant decrease in both the protein mass (Figure 3) and the abundance of mRNA encoding this kinase (Figure 4). This decrease in kinase activity appears to be responsible for the increased activity of BCKDH in livers of clofibrate-treated rats.

In contrast to the liver, clofibrate treatment did not affect the BCKDH kinase activity (Figure 5), its protein mass (Figure 6), or the abundance of mRNA encoding this kinase (Figure 7) in skeletal muscle. The difference between the effect of clofibrate treatment in the liver and skeletal muscle may be related to the levels of clofibrate in these two tissues. In rats receiving clofibrate, the concentration of clofibrate is nearly 3-fold higher in the liver than in skeletal muscle [41].

In conclusion, the results of the present study and our recent studies bring a new dimension to the current concept regarding the regulation of catabolism of branched-chain amino acids. Our recent study in primary cultured hepatocytes showed up-regulation of gene expression of BCKDH [11]. The present study showed down-regulation of gene expression of BCKDH kinase. Therefore, changes in the transcription and translation of the two key enzymes have to be considered as possible mechanisms of alteration in branched-chain amino acid catabolism in various metabolic and nutritional states.

This work was supported by Research Grant AM-15855 from the National Institutes of Health. We thank Dr. Sidney Morris, Jr. for many helpful discussions.

REFERENCES

1 Paul, H. S. and Adibi, S. A. (1984) in Branched-Chain Amino and Keto Acids in Health and Disease (Adibi, S. A., Fekl, W., Langenbeck, U. and Schauder, P., eds.), pp. 182–219, S. Karger AG, Basel
2 Harper, A. E., Miller, R. H. and Block, K. P. (1984) Annu. Rev. Nutr. **4**, 409–454
3 Pettit, F. H., Yeaman, S. J. and Reed, L. J. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 4881–4885
4 Paxton, R. and Harris, R. A. (1982) J. Biol. Chem. **257**, 14433–14439
5 Heffelfinger, S. C., Sewell, E. T. and Danner, D. J. (1983) Biochemistry **22**, 5519–5522
6 Griffin, T. A., Lau, K. S. and Chuang, D. T. (1988) J. Biol. Chem. **263**, 14008–14014
7 Yeaman, S. J. (1989) Biochem. J. **257**, 625–632
8 Reed, L. J., Damuni, Z. and Merryfield, M. L. (1985) Curr. Top. Cell Regul. **27**, 41–49
9 Carothers, D. J., Pons, G. and Patel, M. S. (1989) Arch. Biochem. Biophys. **268**, 409–425
10 Miller, R. H., Eisenstein, R. S. and Harper, A. E. (1988) J. Biol. Chem. **263**, 3454–3461

11 Chicco, A. G., Adibi, S. A., Liu, W. Q., Morris, S. M. and Paul, H. S. (1994) J. Biol. Chem. **269**, 19427–19434
12 Chinsky, J. M., Bohlen, L. M. and Costeas, P. A. (1994) FASEB J. **8**, 114–120
13 Zhao, Y., Popov, K. M., Shimomura, Y., Kedishvili, N. Y., Jaskiewicz, J., Kuntz, M. J., Kain, J., Zhang, B. and Harris, R. A. (1994) Arch. Biochem. Biophys. **308**, 446–453
14 Ono, K., Shioya, H., Hakozaki, M., Honda, K., Mori, T. and Kochi, H. (1990) Biochem. Biophys. Res. Commun. **172**, 243–248
15 Zhao, Y., Jaskiewicz, J. and Harris, R. A. (1992) Biochem. J. **285**, 167–172
16 Rudiger, W., Langenbeck, U. and Goedde, H. W. (1972) Biochem. J. **126**, 445–446
17 Shimomura, Y., Nanaumi, N., Suzuki, M., Popov, K. M. and Harris, R. A. (1990) Arch. Biochem. Biophys. **283**, 293–299
18 Brandt, H., Capulong, Z. L. and Lee, E. Y. C. (1975) J. Biol. Chem. **250**, 8038–8044
19 Gillim, S. E., Paxton, R., Cook, G. A. and Harris, R. A. (1983) Biochem. Biophys. Res. Commun. **111**, 74–81
20 Bagchi, S., Wise, L. S., Brown, M. L., Bregman, D., Sul, H. S. and Rubin, C. S. (1987) J. Biol. Chem. **262**, 1558–1565
21 Fort, P., Marty, L., Piechaczyk, M., El Sabrouty, S., Dani, C., Jeanteur, P. and Blanchard, J. M. (1985) Nucleic Acids Res. **13**, 1431–1442
22 Johnson, D. and Lardy, H. (1967) Methods Enzymol. **10**, 94–96
23 Paul, H. S. and Adibi, S. A. (1982) J. Biol. Chem. **257**, 4875–4881
24 Goodwin, G. W., Zhang, B., Paxton, R. and Harris, R. A. (1988) Methods Enzymol. **166**, 189–201
25 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
26 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
27 Nebes, V. L. and Morris, S. M. (1988) Mol. Endocrinol. **2**, 444–451
28 Feinberg, A. P. and Fogelstein, B. (1983) Anal. Biochem. **132**, 6–13
29 Laskey, R. A. and Mills, A. D. (1975) Eur. J. Biochem. **56**, 335–341
30 Popov, K. M., Shimomura, Y. and Harris, R. A. (1991) Protein Expression Purif. **2**, 278–286
31 Paul, H. S. and Adibi, S. A. (1992) J. Biol. Chem. **267**, 11208–11214
32 Lazarow, P. B. (1981) Methods Enzymol. **72**, 315–319
33 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
34 Snedecor, G. W. (1955) Statistical Methods, 4th edn., pp. 54–72, Iowa State University Press, Ames, IA
35 Paul, H. S., Sekas, G. and Winters, S. J. (1994) Metabolism **43**, 168–173
36 Stanko, R. T., Sekas, G., Isaacson, I. A., Clarke, M. R., Billiar, T. R. and Paul, H. S. (1995) Metabolism **44**, 166–171
37 Lazarow, P. and de Duve, C. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2042–2046
38 Lock, E. A., Mitchell, A. M. and Elcombe, C. R. (1989) Annu. Rev. Pharmacol. Toxicol. **29**, 145–163
39 Paul, H. S. and Adibi, S. A. (1980) J. Clin. Invest. **65**, 1285–1293
40 Voltti, H., Savolainen, M. J., Jauhonen, V. P. and Hassinen, I. E. (1979) Biochem. J. **182**, 95–102
41 Baldwin, J. R., Witiak, D. T. and Feller, D. R. (1980) Biochem. Pharmacol. **29**, 3143–3154

Received 30 October 1995/5 February 1996; accepted 19 March 1996