Longer-term regulation of branched-chain-2-oxoacid dehydrogenase complex studied in rat hepatocytes in culture

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The effect of protein-free diet to decrease liver activity of branched-chain (-2-oxoacid) dehydrogenase (BCD) complex (active form) and increase BCD kinase activity was unaffected by preparation of hepatocytes, but partially reversed by 25 h of culture of hepatocytes in medium 199. Activation of BCD complex preceded loss of BCD kinase. The effect of culture on BCD complex was completely prevented by omission of branched-chain amino acids and partially prevented by 1 mM- α -cyano-4-hydroxycinnamate or 0.2 mM-pyruvate/2 mM-lactate. Protein-free diet decreased plasma branched-chain amino and oxo ('keto') acids and increased plasma pyruvate and lactate. It is concluded: (1) that branched-chain amino acids are involved directly in regulation of activities of BCD complex and BCD kinase; (2) that mitochondrial uptake of branched-chain oxo acids is necessary for regulation of BCD complex activity; and (3) that the stable increase in BCD kinase may function as a hysteresis mechanism.

INTRODUCTION

The mitochondrial branched-chain-2-oxoacid dehydrogenase (BCD) complex of animal tissues is regulated by reversible phosphorylation catalysed by BCD kinase and BCD phosphatase [1–4]. Phosphorylation is inactivating. In rats the activity state of BCDC (percentage of BCDC in the active form) in liver is dependent on the protein content of the diet [5–7]. Thus, for example, when rats maintained on 17%-protein chow are switched to protein-free diet, the activity state of BCDC in liver is decreased from 50 to 3.5% over 10 days [7, 8]. Protein-free diet also leads to a stable 4-fold increase in the activity of BCDC kinase [9]. This increase is referred to as stable because it is demonstrable in extracts of mitochondria and in BCDC purified by gel filtration or fractional precipitation [9]. A study of the time course of these changes in response to protein-free diet showed that the decrease in activity state of BCDC was substantially complete in 4 days, whereas the increase in BCD kinase took 10 days to reach a maximum. On refeeding 17%-protein chow, complete reversal took 20–30 days, but the activity state of BCDC increased from 4 to 27% in 24 h and the activity of BCD kinase fell by 51% in 24 h. There was evidence to suggest that the time course for change in activity state of BCDC did not parallel that for activity of BCD kinase.

There is a need for an '*in vitro*' model in which longer-term regulation of BCDC may be studied in defined media. The present study shows that cultured primary hepatocytes may provide such a model. Tissue culture of hepatocytes from livers of rats fed 17%-protein chow or protein-free diets has been used to investigate some of the factors that may mediate effects of protein-free diet and of the reversion when protein feeding is resumed.

EXPERIMENTAL

Materials and rats

Sources of chemicals, diets and rats were as in [8–10]. Collagenase and α -cyano-4-hydroxyacinnamic acid were from Sigma Chemical Co., Poole, Dorset, U.K.; enzymes for assay of serum metabolites were from BCL, Lewes, East Sussex, U.K. Sources of Falcon tissue-culture bottles, medium 199, foetal-calf serum and antibodies were as in [11]. Medium 199 contained 5.5 mM-glucose, 0.6 mM-sodium acetate, 0.91 mM-DL-leucine, 0.3 mM-DL-isoleucine and 0.46 mM-DL-valine (for other aspects of composition see [12]). BCDC was purified from ox kidney [2] and pyruvate dehydrogenase complex from pig hearts [13].

Hepatocytes and culture

Hepatocytes were from livers of 300–350 g rats; general details of preparation and culture were as in [11]. Viability of cells before culture (as measured by Trypan Blue exclusion) was > 80%; viability of attached cells after culture was > 95%. Culture was for 25 h in medium 199 with changes of medium at 4 and 21 h; foetal-calf serum (5%, v/v) was present from 0 to 4 h. Other additions were from 0 to 25 h. Cells were plated at a density of 10^6 cells/ml (27 ml/flask). A single liver provided hepatocytes for 10–12 flasks. Recovery of cells after culture was determined by assays of cell citrate synthase at 0 and 25 h.

Mitochondria and assays

Mitochondria were isolated as in [11]. Briefly, the flasks were washed vigorously with sucrose medium to remove unattached cells, and attached cells were then removed into fresh sucrose medium with a rubber-coated spatula.

Abbreviations used: BCD(C), branched-chain-2-oxoacid dehydrogenase (complex); ketoleucine, 4-methyl-2-oxopentanoate; ketoisoleucine, 3-methyl-2-oxopentanoate; ketovaline, 3-methylbutyrate; DCA, sodium dichloroacetate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

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Cells were disrupted with a Teflon/glass Potter–Elvehjem homogenizer with re-extraction of the pellet (twice). Mitochondria were isolated by centrifugation and resuspended and centrifuged once. For assay of BCDC (active form), mitochondria were prepared in sucrose medium containing 1 mM-dichloroacetate (DCA) and 100 mM-NaF, and the final pellet frozen in liquid N₂. For assay of total BCDC (sum of active and inactive forms), mitochondria were prepared in sucrose medium (no additions) and incubated in KCl medium containing 10 μ M-CCCP and 1 mM-DCA to effect conversion of inactive complex into active complex as in [7], centrifuged, and pellets frozen in liquid N₂. Extracts were prepared for assay of BCDC protein and citrate synthase or BCD kinase, and assays were performed as in [9].

Blood serum and assays

Blood (10 ml) was withdrawn from the inferior vena cava under Sagatal anaesthesia with a syringe containing 25 μ l of 0.5 M-EDTA to prevent clotting. Plasma was separated after centrifugation, and ultrafiltrates (0.4 ml/ml of plasma) prepared in a MPS1 ultrafiltration apparatus with YMT membranes (Amicon Ltd, Stonehouse, Glos., U.K.). L-Lactate, and branched-chain amino acids (total) were assayed spectrophotometrically by enzymic methods given respectively in [14] and [15]. Samples of ultrafiltrate for assay of branched-chain amino acids were dried down in the cuvette by vacuum desiccation, taken up in 250 μ l of assay mix, incubated for 1 h at 30 °C to complete the reaction, and then diluted with assay mix to 1.4 ml and the A_{340} measured (because of the high K_m of leucine dehydrogenase [16]). Pyruvate and branched-chain oxo acids were assayed successively with pyruvate dehydrogenase and BCDC complexes [16]. This assay may measure ketoleucine, ketoisoleucine, ketovaline, 2-oxobutyrates and the transamination product of methionine [23].

RESULTS AND DISCUSSION

Hepatocytes and culture

The yields of hepatocytes from livers of rats fed chow diet (17% protein) or protein-free diet were 374 ± 30 (28) and 297 ± 13 (49) respectively [mean (millions of cells) $\pm$ S.E.M. with the number of observations in parentheses; $P < 0.02$ for difference]. The decrease in yield due to a protein-free diet (20%) corresponds closely to the decrease in hepatic citrate synthase induced by protein-free diet (22%) [7] and presumably reflects a decrease in hepatocyte mass in the liver. The viabilities at 0 h (79.5 and 78.2%) and after 25 h of culture (97 and 96%) and the recovery of hepatocytes after 25 h of culture (50 and 52%) did not differ significantly.

Preparation and culture of hepatocytes had no effect on mitochondrial activities of citrate synthase or total BCDC for livers of rats on either diet. Citrate synthase activities [munits/mg of mitochondrial protein; mean $\pm$ S.E.M.; (number of observations)] were: chow diet, 171 ± 15 (8) (hepatocytes, 25 h culture) and 206 ± 7 (4) (livers); protein-free diet, 184 ± 8 (11) (hepatocytes, 25 h culture) and 198 ± 6 (6) (livers). Likewise preparation and culture of hepatocytes had no effect on total BCDC activities: chow diet, 13.4 ± 0.4 (40) (livers, taken from [7]); 11.6 ± 1.2 (10) (hepatocytes before culture) 11.9 ± 0.4 (12) (hepatocytes, 25 h culture); protein-free diet, 13.8 ± 0.5 (15) (livers, taken from [7]); 12.4 ± 0.6 (28)

(hepatocytes before culture), 11.9 ± 0.8 (18) (hepatocytes, 25 h culture). These values for total BCDC were adjusted to a mitochondrial citrate synthase activity of 250 munits/mg of protein by the formula given in [7].

Fig. 1. Effect of dietary protein content on concentrations in blood plasma ultrafiltrates and on the activity state of BCDC and the activity of BCD kinase in the rat liver

Rats were fed protein-free diets for up to 10 days and re-fed 17%-protein (chow) diet for 1 or 30 days as shown. At the times shown, rats were anaesthetized with Sagatal, and blood collected from the inferior vena cava, plasma separated and ultrafiltrates prepared and assays performed (see the Experimental section). A chow-fed control (day 0) was included in each experiment and values normalized against the overall mean for controls. Results are means for 11 animals (chow-fed controls, zero time) or for three to ten animals (all other points). Levels of significance (t test) were: branched-chain oxo acids, $P < 0.02$ at 1 and 10 days of protein-free diet and 1 day of refeeding chow diet; branched-chain amino acids and lactate, $P < 0.02$ at 1, 5 and 10 days of protein-free diet and 1 day of refeeding chow diet; pyruvate, $P < 0.02$ at 1 and 10 days of protein-free diet. For other differences, $P > 0.05$. The values for activity state of BCDC and activity of BCD kinase are taken from a previous study [8] and are included for comparison.

Diet and concentrations of metabolites in blood plasma ultrafiltrates

Fig. 1 shows the time course of changes in plasma ultrafiltrate concentrations of branched-chain oxo acids, branched-chain amino acids, pyruvate and lactate induced by protein free-diet and the reversion on refeeding a 17%-protein (chow) diet. Values for the activity state of BCDC and for BCD kinase, which were necessarily measured in a separate study [8], are included for comparison. Branched-chain oxo acids are inhibitors of BCD kinase [17]; lactate plus pyruvate has been shown by Harris *et al.* [18] to decrease the activity state of BCDC in rat hepatocytes in short-term incubations, albeit at higher than physiological concentrations.

Protein-free diet decreased concentrations of branched-chain oxo and amino acids by ~ 50 % and increased lactate and pyruvate by 2–2.5-fold. These changes reversed on feeding chow diet for 30 days. The activity state of BCDC complex fell to a near minimum value over 4 days, whereas BCD kinase activity began to increase on day 2, with the major increase from days 5 to 10 of protein-free diet. On refeeding chow diet, substantial reversion occurred within 24 h, with complete reversion over 30 days. The results (Fig. 1) indicate that changes in circulating branched-chain oxo and amino acids, lactate and pyruvate may be correlated with changes in activity state of BCDC over the first 24–48 h of feeding protein-free diet and on refeeding chow diet. The changes in BCD kinase activity were correlated inversely with activity state of BCDC mainly in the reversion induced by refeeding chow diet.

These results suggested that tissue culture might be useful to investigate the possible influence of branched-chain oxo and amino acids, lactate and pyruvate on the activity state of BCDC and to investigate the correlation between activity state of BCDC and the activity of BCD kinase. Tissue culture has the advantage that the medium composition is defined, and possible influences of other factors such as hormones are excluded.

Activity state of BCDC and activity of BCD kinase in cultured hepatocytes

As shown in Table 1, column 4, lines 1 and 3, the activity state of BCDC in freshly prepared hepatocytes (chow or protein-free diets) did not differ significantly from that in liver (49 %, chow diet; 3.5 %, protein-free diet [7]). Likewise activities of BCD kinase (Table 1, column 5, lines 1 and 3) were comparable with those in liver (1.32 min⁻¹, chow diet; 5.02 min⁻¹, protein-free diet [9]).

After 25 h of culture in medium 199, the activity state of BCDC increased by 25 % (chow-fed) and ~ 5-fold (protein-free diet); the activities of BCD kinase fell by 31 % and by 58 % (Table 1, lines 1 versus 2, 3 versus 4). The increase in activity state of BCDC (protein-free diet) was unchanged when Falcon Primaria tissue-culture flasks were used in place of foetal-calf serum for cell attachment (results not shown). Fig. 2 shows the time course of these changes in hepatocytes (rats fed protein-free diet) cultured in medium 199. The activity state of BCDC increased ~ 4-fold within 8 h, whereas the decline in activity of BCD kinase occurred from 12 to 25 h, i.e. the reversion in activity state was largely independent of the activity of BCD kinase as assayed in mitochondrial extracts. This does not of course preclude changes in the activity of BCD kinase, which could be induced *in vivo* through altered concentrations of effectors of BCD kinase.

The total concentration of branched-chain L-amino acids in medium 199 (assuming 0.5 times the concentration of DL-amino acids) is 0.84 mM, i.e. approximately two or three times the concentration in plasma of rats fed chow or protein-free diets respectively (Fig. 1; see also Suzic *et al.* [19]). Thus the increase in activity state of BCDC induced by 25 h of culture (chow-fed rats) might be due to inhibition of BCD kinase by branched-chain oxo acids formed within hepatocytes by deamination. Culture of hepatocytes for 25 h in modified medium 199 lacking branched-chain amino acids decreased the

Table 1. Activity state of BCDC and activity of BCD kinase in cultured rat hepatocytes

Hepatocytes were isolated from the livers of rats fed 17%-protein chow diet or protein-free diet as shown and cultured for the period shown in medium 199 with variations as shown. Cells were harvested, disrupted and mitochondria isolated, processed, and extracts prepared and assayed for BCDC (total and active form) and BCD kinase. Appropriate controls were incorporated in each culture and results normalized against the mean control. For further details, see the Experimental section. **P* < 0.01, †*P* < 0.02 and ‡*P* < 0.05 for difference from 0 h culture; §*P* < 0.01 and ||*P* < 0.05 for effect of diet; ¶*P* < 0.01 and ***P* < 0.05 for effect of culture medium. The number of hepatocyte preparations is shown in parentheses. Abbreviation: 17 % chow, 17 %-protein chow.

Variations to culture medium 199	Culture time (h)	Diet	Activities (mean ± S.E.M.) for:	
			BCDC (% in active form)	BCD kinase (min ⁻¹)
None	0	17 % Chow	46.5 ± 2.6 (4)	0.91 ± 0.15 (3)
	25	17 % Chow	57.9 ± 4.8 (4)‡	0.63 ± 0.03 (3)†
	0	Protein-free	6.1 ± 2.2 (9)§	4.80 ± 0.37 (18)§
	25	Protein free	30.0 ± 8.0 (5)†	2.03 ± 0.66 (3)*
None	25	17 % Chow	49.7 ± 2.5 (18)	
Free of branched-chain amino acids	25	17 % Chow	26.0 ± 4.9 (4)**	
0.2 mM-Pyruvate/2 mM-lactate	25	17 % Chow	34.1 ± 4.3 (7)¶	
None	25	Protein-free	24.9 ± 1.9 (27)§	
Free of branched-chain amino acids	25	Protein-free	10.4 ± 0.9 (4)§¶	
0.2 mM-Pyruvate/2 mM-lactate	25	Protein-free	11.5 ± 1.9 (8)§¶	
α-Cyano-4-hydroxycinnamic acid (1 mM)	25	Protein-free	15.2 ± 2.4 (4)**

Fig. 2. Activity state of BCDC (●) and activity of BCD kinase (○) in cultured hepatocytes

Hepatocytes were isolated from livers of rats fed protein-free diet for 10 days and cultured in medium 199 for up to 25 h. At the times shown the hepatocytes were harvested and mitochondria isolated and processed for assays of BCDC (total, active form) and BCD kinase (see the Experimental section). A zero-time control was included in each batch of cultures and results normalized against the mean control. Points are means for nine hepatocyte preparations (BCDC activity state, zero time), 18 hepatocyte preparations (BCD kinase, zero time) and three to five preparations (other points). Levels of significance (*t* test) were: BCDC activity state, $P < 0.02$ at 8 and 25 h for difference from zero time; BCD kinase, $P < 0.01$ at 16 and 25 h for differences from zero time and 12 h. For other differences from zero time, $P > 0.05$.

activity state of BCDC to 26 % (chow diet) and to 10.4 % (protein-free diet) (Table 1, lines 6 and 9 respectively). Thus absence of branched-chain amino acids maintained the low activity state of BCDC during culture of hepatocytes from rats fed a protein-free diet and decreased the activity state during culture of hepatocytes from rats fed chow diet.

Evidence that effects of branched-chain amino acids on the activity state of BCDC are mediated by branched-chain oxo acids was obtained with α -cyano-4-hydroxycinnamate. This compound was shown by Halestrap [20] and by Mackay & Robinson [21] to be a good inhibitor of rat liver mitochondrial transport of ketoleucine (K_i 6.3 μ M [20]) and a less effective inhibitor of transport of ketoisoleucine (K_i 42 μ M [21]) and ketovaline (K_i 133 μ M [21]). Ketoleucine and ketoisoleucine are more effective inhibitors of BCD kinase than ketovaline [17]. As shown in Table 1 (line 11), 1 mM- α -cyano-4-hydroxycinnamate inhibited by 46 % the increase in activity state occasioned by culture of hepatocytes (protein-free diet) in medium 199. Patel *et al.* [22] found in rat liver mitochondria a maximum of 60 % inhibition of decarboxylation of [1-¹⁴C]ketoleucine or of [1-¹⁴C]ketovaline by the related inhibitor α -cyanocinnamate at concentrations up to 2 mM, a comparable extent of inhibition in perfused rat liver (ketovaline), but no inhibition of decarboxylation in

mitochondrial extracts. Thus their findings may indicate that α -cyanocinnamate inhibition of uptake of branched-chain oxo acids by liver mitochondria is incomplete. This might explain why reversal of the activity state of BCDC by the 4-hydroxy derivative was incomplete in our experiments.

As shown in Table 1 (lines 7 and 10), pyruvate (0.2 mM) plus lactate (2 mM) in medium 199 at near circulating concentrations (see Fig. 1) decreased the activity state of BCDC in hepatocytes cultured for 25 h by 31 % (chow diet) or 54 % (protein-free diet). The mechanism of action is not known with certainty, but there is evidence that ketoleucine may be transported in rat liver mitochondria by the pyruvate carrier [20]. Thus pyruvate might inhibit mitochondrial uptake of branched-chain oxo acids and thereby increase the activity of BCD kinase.

CONCLUSIONS

A major function of regulation of the activity state of BCDC by reversible phosphorylation is to adjust the rate of degradation of branched-chain amino acids in relation to dietary intake. In the rat this regulation is predominantly hepatic [24]. The effects of dietary intake of protein on the activity state of rat liver BCDC are slow and occur over several days [5,7,10]. There is a need, therefore, for an '*in vitro*' model to study the long-term effects of nutrients and hormones in defined media on regulation of BCDC in liver by reversible phosphorylation. The present study has shown that primary hepatocytes in culture are a suitable model for at least 25 h (the longest period tested).

With this system it has been shown that the decrease in activity state of BCDC, and the stable increase in BCD kinase activity induced by protein-free diet *in vivo*, are partially reversed by 25 h of culture in medium 199. Thus the ratio (chow-fed/protein-free diet) for activity state of BCDC was decreased by 25 h of culture from 7.6 to 1.9 and that for BCD kinase was increased from 0.19 to 0.31. The effect of 25 h of culture (hepatocytes from rats fed protein-free diet) on the activity state of BCDC and the activity of BCD kinase were strictly comparable with those induced *in vivo* by 24 h of refeeding chow diet. It has been shown that these effects of culture on activity state require branched-chain amino acids and that the effect of branched-chain amino acids is partially blocked by a known inhibitor of mitochondrial uptake of branched-chain ketoacids, namely α -cyano-4-hydroxycinnamate. Therefore the decreased circulating concentrations of branched-chain amino and oxo acids on protein-free diet may be one factor decreasing the activity state of hepatic BCDC. This could be mediated directly through known regulation of BCD kinase by branched-chain oxo acids.

The observation by Harris *et al.* [18] that lactate plus pyruvate decrease the activity state of BCDC in hepatocytes has been shown in the present study to occur also in longer-term culture and at physiological concentrations. It has been shown also that protein-free diets increase circulating lactate and pyruvate concentrations *in vivo*, with reversion on refeeding chow diet. Least-squares linear-regression analysis of the relationship (data of Fig. 1) between [plasma ultrafilterable pyruvate]⁻¹ and activity state of BCDC in liver *in vivo* showed a linear relationship ($P < 0.01$ for linear regression; cor-

relation coefficient, $r = 0.968$; $P < 0.001$); i.e. there was a highly significant inverse correlation between plasma ultrafilterable pyruvate and the activity state of BCDC in liver.

It has been shown in a previous study [9] that protein-free diet induces a stable 4-fold increase in the activity of BCD kinase in liver *in vivo*. A study of the time course [8] showed that 70 % of the increase in BCD kinase activity occurred after inactivation of BCDC by phosphorylation was essentially complete. It was not possible to tell from the results of that study [8] whether the two parameters were dissociated during the reversion induced by feeding 17%-protein chow diet. In the present study reversion was monitored in culture, and it was shown that substantial re-activation of BCDC occurs before any detectable decrease in the activity of BCD kinase. These observations suggest that the stable increase in BCD kinase activity induced by protein-free diet may not be the major factor decreasing the activity of BCDC by phosphorylation. It is suggested that the stable increase in BCD kinase activity may function mainly as a hysteresis mechanism. Thus it may limit the extent of re-activation of BCDC when protein intake is resumed after protein deprivation. This might have the effect of directing absorbed branched-chain amino acids towards protein synthesis.

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