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Gender difference in regulation of branched-chain amino acid catabolism

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Regulation of the activity state of the hepatic branched-chain 2-oxo acid dehydrogenase (BCODH) complex during the light–dark cycle differs markedly in male and female rats. Female rats exhibit a profound diurnal rhythm in the activity state of the complex that is not observed in male rats. Regardless of gender, most of the complex was dephosphorylated and active in the middle of the dark period and early in the light period, and this form of the complex predominated in male rats at the end of the light period. In contrast, most of the complex in female rats became phosphorylated and inactive by the end of the light period. Gonadectomy prevented the diurnal rhythm in females but was without effect in males, indicating that female sex hormones are required for this gender difference in regulation of the BCODH complex. Changes in levels of branched-chain 2-oxo acids, known regulators of BCODH kinase, do not seem to be involved; rather, an increase in BCODH kinase activity occurring between morning and evening is responsible for inactivation of the BCODH complex in female rats. The increase in kinase activity is due to an increase in the amount of kinase protein associated with the BCODH complex. Thus a marked diurnal variation in the amount of BCODH kinase and therefore its activity results in large swings in the activity state of the liver BCODH complex in female rats. This study provides the first evidence for a gender-specific difference in the regulation of branched-chain amino acid catabolism.

INTRODUCTION

The branched-chain 2-oxo acid dehydrogenase (BCODH) complex, the most important regulatory enzyme of branched-chain amino acid catabolism, is expressed at exceptionally high levels in rat liver (reviewed in [1–3]). The BCODH complex functions in this tissue as a sink for the disposal of extra circulating branched-chain 2-oxo acids derived from the transamination of leucine, isoleucine and valine in peripheral tissues. This enzyme is maintained in its active, dephosphorylated state in the liver under conditions where branched-chain amino acids and their 2-oxo acids are present in excess [1–3]. Irreversible disposal of these amino acids and 2-oxo acids is important because of their toxicity, well established by clinical experience with the genetic disease known as maple syrup urine disease. In contrast, turning off this enzyme by phosphorylation of its E1 component by a specific kinase (BCODH kinase) is important for the conservation of the branched-chain 2-oxo acids and therefore the branched-chain amino acids during times of dietary insufficiency. The branched-chain amino acids cannot be synthesized in the body but are essential for protein synthesis; they must therefore be supplied by the diet and also conserved when dietary supplies are inadequate. Thus a tight regulation of the hepatic BCODH complex is of critical importance when branched-chain amino acids are in short supply or present in excess amounts.

Practically all of the data in support of the above model of the role of the BCODH complex in branched-chain amino acid catabolism have been obtained with rapidly growing male rats. Virtually no studies of the regulation of branched-chain amino acid catabolism have been done with female rats. Sexually mature female rats grow more slowly and have a lower feed efficiency (ratio of body weight gain to food consumption) than male rats. Because it seemed likely that the gender difference in growth might affect branched-chain amino acid metabolism, a study of the regulation of the hepatic BCODH complex in female rats was initiated. We report here the first evidence for a marked difference in the regulation of branched-chain amino acid catabolism in female rats. A diurnal variation in the amount of BCODH kinase, and therefore its activity, seems to be an important factor. Findings with surgically modified male and female rats suggest that female sex steroids are involved.

MATERIALS AND METHODS

Materials

Reagents were obtained as previously described [4,5] or from Sigma Chemical Company. Male and female Sprague–Dawley rats (7 weeks old) were obtained from CLEA Japan (Tokyo, Japan). A semipurified diet containing 50 % protein was purchased from CLEA Japan and consisted of the following ingredients in percentage by weight: AIN 76 mineral mix, 3.5; AIN 76 vitamin mix 1; soybean oil, 5; choline bitartrate, 0.2; D,L-methionine, 0.3; inositol, 0.01; cellulose, 5; milk casein, 50; corn starch, 20; and sucrose, 15.0.

Extraction and assay of BCODH complex and BCODH kinase

Extraction of the BCODH complex from rat liver was performed as described previously [5]. The activities of the BCODH complex in the active form (actual activity) and in the totally dephosphorylated form (total activity) were determined spectrophotometrically by the rate of NADH production as described previously [6]. One unit of the complex catalysed the formation

Abbreviation used: BCODH, branched-chain 2-oxo acid dehydrogenase.

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of 1 μmol of NADH/min. The activity state of the complex was calculated as a percentage by multiplying the ratio of the actual activity to the total activity by 100.

The assay of BCODH kinase was performed as described previously [7] with a modification, described below, in the composition of the kinase resuspension buffer. Liver extracts containing the BCODH complex and its kinase were prepared by the procedure described previously [5]. Dichloroacetate and thiamine pyrophosphate (kinase inhibitors) were omitted from extraction and resuspension buffers. The complex was completely activated by dephosphorylation with a broad-specificity phosphoprotein phosphatase and then precipitated again with 9 % (w/v) poly(ethylene glycol). The precipitate was resuspended in a kinase buffer consisting of 20 mM Hepes, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, 25 mM potassium phosphate, 50 mM KF, 25 μg/ml oligomycin and 20 % (v/v) glycerol, pH 7.5. Reactions were initiated by addition of 0.5 mM ATP. Incubations were performed at 30 °C and samples (8 m-units of the complex) were taken at specified time points (0, 30, 60, 90 and 120 s) for assay of the remaining BCODH complex activity. Apparent first-order rate constants for dehydrogenase inactivation were calculated by least-squares linear regression analysis.

Western blot analysis

The BCODH complex and its associated kinase were isolated from rat liver by immunoaffinity chromatography with goat anti-(BOCDH complex) IgG coupled to Sepharose 4B [8]. The amount of BCODH kinase associated with the immunoaffinity-purified complex was quantified by Western blot analysis as described previously [8].

Determination of branched-chain 2-oxo acids in plasma

Blood was withdrawn into heparinized syringes by cardiac puncture under ether anaesthesia. Plasma was collected by centrifugation at 1000 ***g*** for 5 min. Total branched-chain 2-oxo acids were measured spectrophotometrically by the procedure described by Goodwin et al. [9].

Animal care and experimental design

Rats were individually caged in a well-ventilated animal room (temperature 24 ± 2 °C; artificial lights turned on from 05:00h to 17:00h; complete darkness from 17:00h to 05:00h) and fed *ad libitum* with a chow diet (CE-2; CLEA Japan; protein content approx. 25 %) for 1 week before experiments. The light cycle of the animal room used in the U.S.A. was 07:00h–19:00h and rats were fed on Purina Rodent Laboratory Chow 7001 (protein content approx. 23 %). The mean body weight of 8-week-old rats was approx. 230 g for males and approx. 170 g for females.

Expt. 1 was designed to compare the activities of the hepatic BCODH complex and its kinase in male and female rats fed chow and a high protein diet. Male ($n = 13$) and female ($n = 12$) rats (8 weeks old) were randomly divided into two groups which were fed with chow and 50 % protein diets respectively. Diets were given in a powder form *ad libitum* for 3 weeks. On the final day of the experiment, rats were deprived of food for approx. 7 h before being killed between 16:00h and 17:00h. After cervical dislocation of the rats, livers were rapidly removed (within 50 s), freeze-clamped at liquid nitrogen temperature and stored at −80 °C until analysed.

Expt. 2 was designed to investigate diurnal changes in the liver BCODH complex activity in male and female rats. Rats 9 weeks old (11 males and 12 females) were fed with the 50 % protein diet *ad libitum* for 2 weeks. Food intake was measured three times daily (at 09:00h, 17:00h and 01:00h) on the last 2 days of the experiment. Four rats of each group were killed at 01:00h, a time point within the dark period where food consumption would be expected to be maximal (well-fed time point). Four more rats of each group were killed at 09:00h, corresponding to a postprandial time point 4 h after the beginning of the light phase of the day. To provide a post-absorptive time point, the rest of the rats (four males and four females) were deprived of food for an additional 7–8 h before being killed at the end of the light period (17:00h). Procedures for the killing of rats and treatment of livers were as described for Expt. 1.

Expt. 3 determined whether diurnal changes of BCODH kinase activity and protein amount occur in male and female rats. For this experiment rats (six males and six females) were fed on a chow diet for 2 weeks. On the final day of the experiment, rats were killed at two time points: 09:00h (2 h after the beginning of the light phase of the day) and 19:00h (at the end of the light period), and livers were collected as described for Expt. 1.

Expt. 4 was conducted to compare branched-chain 2-oxo acids in the plasma of male and female rats at two time points. Wistar rats (six males and six females) were maintained on a chow diet for 1 week and blood was drawn into a heparinized syringe by cardiac puncture under ether anaesthesia at 09:00h. After 4 days of recovery, blood was collected by the same procedure at 19:00h (at the end of the light period).

Expt. 5 was designed to investigate the effects of gonadectomy on the activities of the liver BCODH complex and its kinase in male and female rats. Rats (11 males and 11 females; 8 weeks of age) were randomly divided into two surgical treatment groups: gonadectomized and sham-operated. For surgery, the animals were anaesthetized with sodium pentobarbital (50 mg/kg body weight, injected intraperitoneally). Males were castrated through an incision in the scrotum; females were ovariectomized through a dorsolateral incision. Care was taken to remove as little non-ovarian tissue as possible. After operation, penicillin and streptomycin (100 i.u. and 100 μg/day respectively) were injected intraperitoneally in saline for 2 days. After surgery, all rats were fed with chow diet *ad libitum* for 3 weeks. The food consumption of operated rats was restored to the normal level within 2 days after surgery. Procedures for the killing of rats and treatment of livers were as described for Expt. 1.

Statistics

Statistical analysis was performed by a 2×2 factorial ANOVA and Scheffé test [10]. Data are presented as means ± S.E.M. Differences with $P < 0.05$ were considered significant.

RESULTS

Activities of the hepatic BCODH complex and its kinase in male and female rats fed with chow and high-protein diets

Rats were fed with either a chow diet or a 50 % semipurified diet for 3 weeks before being killed between 16:00h and 17:00h on the last day of the experiment. As expected from previous findings [11], the weight gain of male rats (167 ± 5 g per 3 weeks on chow diet) was greater than that of the female rats (59 ± 4 g per 3 weeks). Food efficiency (g body weight gain/g food consumed) was likewise greater in males than females (0.20 ± 0.02 compared with 0.08 ± 0.01 respectively). The most remarkable finding was a striking difference between male and female rats in the activity state of the BCODH complex (Table 1): 90 % of the enzyme was in the active, dephosphorylated state in the males, but only 10–15 % of the enzyme was active in the females. The

Table 1 Differences between male and female Sprague–Dawley rats in liver BCODH complex activity and activity state and BCODH kinase activity

Rats were maintained on chow and 50% protein diets for 3 weeks. Values are means ± S.E.M.; *n* is the number of rats. * Significantly different from male rats in the same diet group ($P < 0.05$). BCODH kinase activity is expressed as apparent first-order rate constants for BCODH complex inactivation.

Group	*n*	BCODH complex activity (m-units/g wet wt.) Before activation	After activation	Complex in active state (%)	BCODH kinase (min^{-1})
Chow diet					
Male	6	1669 ± 70	1849 ± 57	90 ± 2	0.25 ± 0.02
Female	5	213 ± 85*	1246 ± 142*	15 ± 5*	1.25 ± 0.18*
50% Protein diet					
Male	7	1334 ± 108	1540 ± 68	87 ± 6	0.39 ± 0.03
Female	7	113 ± 28*	1285 ± 68	9 ± 2*	1.04 ± 0.09*

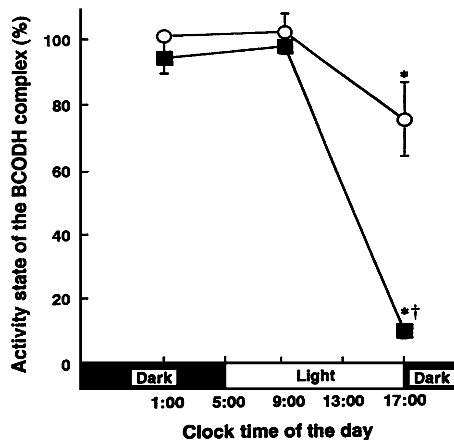

Figure 1 Diurnal change of hepatic BCODH complex activity in rats fed with 50% protein diet

Values are means ± S.E.M. for three or four rats per time point. Symbols: ■, female rats; ○, male rats. * Significantly different from the values at other time points in the same gender ($P < 0.05$); † significantly different from male rats at the same time point ($P < 0.05$). The experiment shown was conducted with Sprague–Dawley rats. Comparable results, not shown, were obtained with Wistar rats.

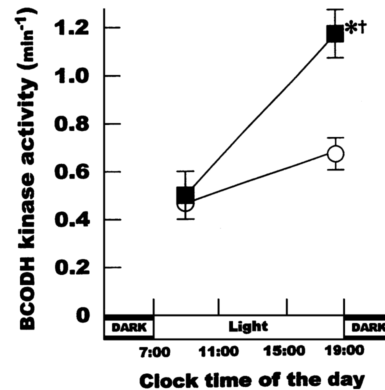

Figure 2 Diurnal change of BCODH kinase activity in Wistar rats fed with chow diet

Values are means ± S.E.M. for three rats per time point. Symbols: ■, female rats; ○, male rats. * Significantly different from the values at other time points in the same gender ($P < 0.05$); † significantly different from male rats at the same time point ($P < 0.05$). Comparable results, not shown, were obtained with Sprague–Dawley rats.

total activity of the liver BCODH complex was significantly greater (30%) in males than females fed with the chow diet (Table 1), but no significant difference in this measurement was found between males and females fed with the 50% protein diet (Table 1). The activity of liver BCODH kinase was 5-fold higher in females than males fed with the chow diet (Table 1), and it was 3-fold higher in females than males fed with the high protein diet (Table 1). Thus an inverse relationship exists between BCODH kinase activity and the activity state of the BCODH complex when male rats are compared with female rats.

Studies of diurnal changes of liver BCODH complex and BCODH kinase activities in male and female rats

Rats were fed with the 50% semipurified diet for 2 weeks before this experiment. Both male and female animals were killed at 01:00h (dark period), 09:00h (early light period) and 17:00h (end of light period). Measurement of food consumption between 17:00h and 01:00h and between 01:00h and 09:00h showed no apparent difference in food intake pattern between male and female rats (males, 4.8 ± 0.3 and 2.4 ± 0.3 g/100 g body weight between 17:00h and 01:00h and 01:00h and 09:00h respectively; females, 5.0 ± 0.3 and 3.1 ± 0.1 g/100 g body weight for the same time periods).

The activity states of the BCODH complex at 01:00h and 09:00h in both males and females were very similar and close to 100% (Figure 1). However, as would be expected from the results of Expt. 1, the activity state of the complex at 17:00h was markedly different. Approx. 75% of the enzyme was in the active, dephosphorylated state in the male rats, compared with only 10% of the enzyme in the female rats. In contrast with these marked differences in activity states, total activity of the BCODH complex did not vary over the course of the day in either male or female rats (results not shown). This study therefore shows that phosphorylation of the complex produced a greater inactivation of the complex in female rats than in male rats during the post-absorption period (from 09:00h to 17:00h).

In contrast with the marked gender difference found in BCODH kinase activity in the experiment given in Table 1, there was no difference in kinase activity between male and female rats killed early in the light period (Figure 2). However, by the end of the light period a marked gender difference in kinase activity was apparent that was consistent with the initial experiment. BCODH kinase activity increased 2.3-fold in the female rats between

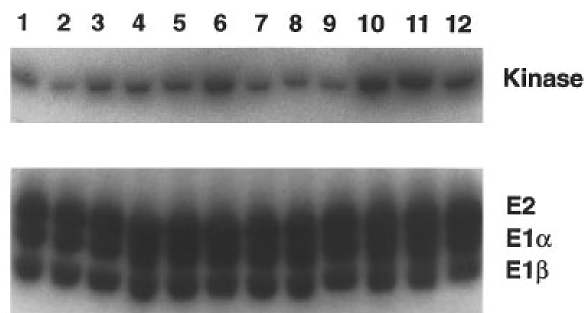

Figure 3 Western blot analysis of BCODH kinase associated with the BCODH complex in male and female Wistar rats

Upper panel, BCODH kinase; lower panel, BCODH subunits (E2, E1α and E1β) to establish that the loading of the BCODH complex was identical for the each group of rats. Lanes 1–3, male rats killed at 09:00h; lanes 4–6, male rats killed at 19:00h; lanes 7–9, female rats killed at 09:00h; lanes 10–12, female rats killed at 19:00h. Comparable results, not shown, were obtained with Sprague–Dawley rats.

morning and evening, whereas a smaller (1.4-fold) increase that was not significantly different occurred in male rats in this time period (Figure 2).

In an effort to determine the basis for the gender difference in kinase activity of rats killed in the evening, the relative amounts of BCODH kinase protein bound to the BCODH complex of male and female rats were compared by Western blot analysis (Figure 3). As expected from the activity measurements (Figure 2), the relative amounts of kinase protein of rats killed in the morning were low and not significantly different between male and female rats (Figure 3). By evening, however, a 2.5-fold increase in BCODH kinase protein associated with the BCODH complex, that can completely explain the increase in enzyme activity, had occurred in the female rats. Although not statistically significant, a modest increase in BCODH kinase protein (1.3-fold) corresponding to a slight increase in BCODH kinase activity occurred in the male rats between morning and evening (Figure 3).

Determination of plasma levels of branched-chain 2-oxo acids in male and female rats

Because branched-chain 2-oxo acids inhibit BCODH kinase activity [12,13], the levels of circulating branched-chain 2-oxo acids were measured in male and female rats at two time points where differences in BCODH activity states were observed. However, no significant differences in concentrations of plasma branched-chain 2-oxo acids between male and female rats were detected: 21.5 ± 2.5 mM in males at 09:00h, 16.7 ± 1.0 mM in males at 19:00h, 15.0 ± 2.0 mM in females at 09:00h, and 19.9 ± 2.0 mM in females at 19:00h. Thus no evidence was obtained in these studies that inhibition of BCODH kinase activity by branched-chain 2-oxo acids is a significant factor in the difference in BCODH complex activity state between male and female rats.

Effects of gonadectomy on the activities of liver BCODH complex and its kinase in male and female rats

Gonadectomized and sham-operated male and female rats were fed with the chow diet for 3 weeks before this experiment. Food intake and body weight gain were not significantly changed by castration of male rats (results not shown). However, ovariectomy greatly increased body weight gain (111 ± 8 compared with 55 ± 5 g per 3 weeks; $P < 0.05$) and food efficiency (0.19 ± 0.01 compared with 0.09 ± 0.01; $P < 0.05$) of female rats, indicating that female hormones suppressed body weight gain and decreased food efficiency in the present study, as expected from previous findings [14,15].

All rats in this experiment were killed at 17:00h to determine the activity state of BCODH complex in the post-absorptive state. Castration of male rats had no effect (Table 2). In contrast, ovariectomy of female rats had the marked effect of increasing the activity state of the BCODH complex from 7% (sham-operated controls) to 100% (Table 2). Thus ovariectomy elevated the activity state of the BCODH complex to that of male rats. Gonadectomy had no effect on total BCODH complex activity in either male or female rats (Table 2). Castration likewise had no effect on BCODH kinase activity in male rats (Table 2), but in female rats ovariectomy decreased BCODH kinase activity nearly to the level of male rats (Table 2).

DISCUSSION

The activities of the liver BCODH complex and its kinase were compared in normal Sprague–Dawley and Wistar male and female rats in the present study. The activity state of the complex at the end of the light period of a day was much lower in female than in male rats. In contrast, liver BCODH kinase activity was much higher in female than in male rats at the end of the light period, a finding that most probably accounts for the gender difference in BCODH complex activity states because this enzyme

Table 2 Effects of gonadectomy on the activity of liver BCODH complex and its kinase in male and female Sprague-Dawley rats

Rats were fed with chow diet for 3 weeks after surgery to remove testes and ovaries. Values are means ± S.E.M. for five or six rats; *n* is the number of rats. * Significantly different from sham-operated male rats ($P < 0.05$). † Significantly different from sham-operated female rats ($P < 0.05$). BCODH kinase activity is expressed as apparent first-order rate constants for BCODH complex inactivation.

Group	*n*	BCODH complex activity (m-units/g wet wt.)		Complex in active state (%)	BCODH kinase (min^{-1})
		Before activation	After activation		
Male					
Sham-operated	5	1876 ± 173	1968 ± 128	95 ± 6	0.18 ± 0.01
Gonadectomized	6	1659 ± 109	1808 ± 123	92 ± 3	0.19 ± 0.01
Female					
Sham-operated	5	98 ± 14*	1360 ± 82*	7 ± 0.8*	1.27 ± 0.28*
Gonadectomized	6	1405 ± 119†	1395 ± 77*	101 ± 7†	0.29 ± 0.02†

is responsible for phosphorylation and inactivation of the complex.

This study clearly demonstrates that the time of day at which animals are killed and tissues collected for determination of activity states of the BCODH complex should be reported. For technical reasons we have routinely killed rats in the late afternoon in Japan and in the early morning in the United States. The gender difference was discovered in Japan and reproduced in the United States once the importance of the time of day at which the rats are killed was realized.

Evidence suggesting a diurnal variation in the activity state of the BCODH complex has been reported previously [16] in a study of male rats fed with a 9% casein diet. However, no evidence has been presented previously for diurnal changes in the activity state of the BCODH complex in rats fed with either adequate or high levels of dietary protein, presumably because previous work has always been done with male rats [1–3,17–21]. Marked differences in activity states of the complex in meal-fed rats before and after consumption of a meal have been reported [21]. For example, the activity state increased from 34% before to 89% after consumption of a meal containing 25% protein. In contrast, the activity state of the BCODH complex of male rats accustomed to eating 50% protein meals was at a high level before and after consumption of a meal containing this high level of protein. Thus changes in BCODH complex activity over the time course of a day can be induced in males by meal feeding, but even this response to feeding and fasting is lost when the diet is rich in protein. In contrast, the present study demonstrated a marked diurnal variation in BCODH activity state in female rats fed with both a high-protein diet and a chow diet *ad libitum*.

It seems very likely that the high BCODH kinase activity found at the end of the light period causes the low activity state of the liver BCODH complex of female rats killed at this time point. This high kinase activity survives dilution of the enzyme for assay and therefore cannot be explained by a decrease in 4-methyl-2-oxopentanoate concentration, the only known inhibitor of this kinase [12,13]. Furthermore changes in plasma levels of branched-chain 2-oxo acid levels that could contribute to the large difference in activity state of the complex between morning and evening were not observed. Rather, the increase in BCODH kinase activity that occurs in female rats at the end of the light cycle was found to be due to an increase in BCODH kinase protein bound to the complex. What mechanism accounts for this increase in kinase protein remains to be determined.

The present study implicates female sex hormones in the regulation of branched-chain amino acid catabolism. Ovariectomy prevented the gender difference in regulation of BCODH complex activity, whereas castration of male rats was without effect, suggesting that female sex hormones are most important in producing the gender difference. Oestradiol induces certain enzymes in liver [22,23], but its effect on BCODH kinase expression has not been studied. Growth hormone is likewise recognized to be responsible for gender differences in the expression of certain hepatic enzymes [24,25], but again no studies on the effect of growth hormone on BCODH kinase expression have been reported. Because liver BCODH complex should dispose of only those branched-chain amino acids in excess of requirements for protein synthesis and therefore growth, a mechanism whereby growth hormone signals inactivation of the complex to conserve branched-chain amino acids for growth would seem desirable. In contrast, it would also seem logical for the BCODH complex to be phosphorylated and inactive in the more rapidly growing male rats as a part of a mechanism designed to conserve branched-chain amino acids for protein synthesis. Interestingly, the opposite is observed, i.e. the more slowly growing, less energy-efficient female rats, which must be using more of their branched-chain amino acids for processes other than protein synthesis and growth, seem to have the hepatic BCODH complex under tighter control. No explanation for this phenomenon can be offered at this time. More work is clearly needed to understand the physiological significance of the gender difference in regulation of branched-chain amino acid metabolism observed in the present study.

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