PRELIMINARY REPORT

Chronic Ethanol Intake Reduces the Flux Through Liver Branched-Chain Keto-acid Dehydrogenase

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Chronic ethanol intake selectively increases concentrations of branched-chain amino acids (BCAA) in the liver. To determine whether a reduced oxidation plays a role in this effect, we measured substrate flux through branched-chain keto-acid (BCKA) dehydrogenase in livers of rats pair-fed liquid diets containing either 0% or 36% of total calories as ethanol for 21 days. Substrate (1.0 mmol/L ketoisocaproate [KIC]) fluxes in the liver of ethanol-fed and control rats were 225 ± 18 and 319 ± 27 μmol/h per whole liver ($P < .05$), respectively. We then studied whether this effect was due to either ethanol or the products of its metabolism, or to an alteration in the activity of BCKA dehydrogenase. Addition of ethanol (25 to 200 mmol/L) to the perfusion medium had no significant effect on the flux through BCKA dehydrogenase in the liver of control rats. Ethanol-fed rats had lower ($P < .01$) basal activity (0.84 ± 0.11 *v* 1.39 ± 0.12 U/g liver) and total activity (0.94 ± 0.11 *v* 1.42 ± 0.11 U/g liver) than control rats, but a similar activity state (90% ± 4% *v* 99% ± 4%) of BCKA dehydrogenase. In conclusion, chronic ethanol intake reduces the flux through liver BCKA dehydrogenase by decreasing the basal and total activity of BCKA dehydrogenase and not increasing the conversion of the enzyme to its inactive form.

CHRONIC ETHANOL INTAKE selectively increases concentrations of branched-chain amino acids (BCAA) in the liver.[1] The mechanism of this metabolic effect of ethanol is not known, but could include inhibition of BCAA catabolism. The liver is the main site of ethanol metabolism, and additionally is a tissue that could appreciably decrease the oxidation of BCAA in response to metabolic perturbations. Branched-chain keto-acid (BCKA) dehydrogenase, the key mitochondrial enzyme regulating the oxidation of BCAA, exists in active (dephosphorylated) and inactive (phosphorylated) forms. The interconversion between the active and inactive forms is catalyzed by a specific phosphatase and a protein kinase, respectively. Normally, BCKA dehydrogenase is almost fully active in the liver, whereas it is only partially active in other tissues.[2] For example, in skeletal muscle, only 1% to 2% of the enzyme is in the active form.[2-4]

In view of these considerations, we investigated whether chronic ethanol consumption alters substrate flux through BCKA dehydrogenase in the liver. Having found that it does, we studied whether the effect was caused by either ethanol or the products of its metabolism, or by an alteration in the activity of BCKA dehydrogenase.

MATERIALS AND METHODS

Animal Treatment

Male Sprague-Dawley rats (100 g) were housed in individual cages in a temperature-controlled room on a 12-hour light/dark cycle and were fed Purina Laboratory Chow (St Louis, MO) until they reached a body weight of 120 to 130 g. The rats were then pair-matched based on weight and fed either control or ethanol-containing liquid diets (41.4 g/L) for 21 days. The composition of the diets and the pair-feeding technique have been previously detailed.[5] The day before the experiments, the daily rat intake was divided into two portions and offered at 9 AM and 7 PM. On the day of experiments, rats received one third of their estimated dietary intake at 7 AM and the diet was removed at 9 AM. Therefore, all animals were in a fed condition at the time of the experiments.

Flux Through Liver BCKA Dehydrogenase

The flux through BCKA dehydrogenase was measured in livers perfused in situ using a single-pass technique and a hemoglobin-free medium as described elsewhere.[6] The perfusion medium was Krebs Henseleit-bicarbonate buffer, pH 7.4, 37°C, and saturated with an O_2/CO_2 mixture (95%/5% vol/vol). The flow rate was 35 mL/min. After a 15-minute equilibration period, sodium ketoisocaproate ([KIC] 1.0 mmol/L) and sodium α-keto[1-^{14}C]isocaproate ([1-^{14}C-KIC] 0.10 μCi/μmol) were added to the perfusion medium at a mixing chamber located immediately before the inlet cannula. Our preliminary experiments, in agreement with another study,[6] showed that 1.0 mmol/L KIC was necessary for the substrate to be unlimited during the flux study. Oxygen consumption by the liver was continuously measured using a flow-through oxygen electrode (Microelectrodes, Londonderry, NH) placed immediately after the liver outflow cannula. Effluent medium was collected at 30-second intervals. Five milliliters of each collection was placed in an Erlenmeyer flask sealed with rubber stoppers equipped with plastic center wells containing 0.3 mL methylbenzethonium hydroxide. $^{14}CO_2$ was released from the perfusion medium by injecting 0.5 mL 5N H_2SO_4 through the rubber stoppers into the flasks. The flasks were agitated for 1 hour before the center wells were transferred to scintillation vials containing 10 mL liquid scintillation fluid and counted by liquid scintillation spectrometry. At the end of the perfusion, a portion of the liver was frozen in metal tongs previously maintained in liquid nitrogen and the rest was weighed and dried to constant weight in an oven at 100°C.

Basal and Total Activity of BCKA Dehydrogenase

Basal and total activities of BCKA dehydrogenase were determined spectrophotometrically as described by Goodwin et al,[2]

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using 0.5 mmol/L α-keto-isovalerate as a substrate. Enzyme sources were obtained from nonperfused livers. Basal activity was measured in the partially purified enzyme preparation without modifications. Total BCKA dehydrogenase activity was measured after incubating an aliquot of the enzyme preparation with partially purified broad-specificity phosphoprotein phosphatase and $MgCl_2$ for 1 hour at 30°C to achieve complete activation of the enzyme. The broad-specificity phosphatase was isolated from rabbit liver as previously described by Paxton and Harris.[7] This preparation releases approximately 90% of ^{32}P-labeled BCKA dehydrogenase by 35 minutes at 37°C.[7] One unit of BCKA dehydrogenase activity represents 1 μmol NADH produced per minute at 30°C.

Statistical Methods

Results are presented as the mean ± SEM. Statistical evaluation of flux rate was performed by repeated-measures ANOVA followed by Scheffe's test.[8] The rest of the data were analyzed using Student's *t* test. All analyses were performed with the BMDP statistical package. *P* values less than .05 were considered significant.

RESULTS

Metabolic Effects of Ethanol

On average, ethanol-treated rats consumed 13.4 ± 0.4 g ethanol/kg/d. We have previously shown[1,5] that rats consuming this amount of ethanol have a plasma ethanol concentration in the toxic range (>25 mmol/L) and significantly increased concentrations of BCAA in the liver. There was no difference between the daily weight gain of control and ethanol-treated rats. As a result, at the time of death, body weights of rats in both groups were similar (222 ± 3 *v* 222 ± 4 g). However, livers of ethanol-treated rat were 18% heavier than those of control rats (10.4 ± 0.9 *v* 8.8 ± 0.3 g, $P < .05$).

Flux Through BCKA Dehydrogenase

To assess viability of the liver during perfusion, we measured oxygen consumption and the wet/dry weight ratio in six livers of each group. Oxygen consumption during perfusion of the liver of control and ethanol-fed rats was 2.72 ± 0.25 and 2.35 ± 0.28 μmol/min/g wet liver (P = NS), respectively. In addition, there was no significant difference between the wet/dry weight ratios before and after perfusion in the livers of control (3.21 ± 0.10 *v* 3.43 ± 0.07) or ethanol-treated (2.52 ± 0.04 *v* 2.51 ± 0.10) rats. These results indicate that viability of the liver was maintained during each perfusion.

The flux was lower in the liver of ethanol-treated rats (41%) as compared with control rats (21.8 ± 1.5 *v* 36.5 ± 3.3 μmol/h/g liver, $P < .01$; Fig 1). It is noteworthy that this reduction in flux could not have been the result of dilution of radiolabeled KIC. Although in the present study we did not measure KIC concentration, in a previous study we found it to be undetectable in the liver.[9] This is due to the low BCAA transaminase activity and high BCAA dehydrogenase activity in the liver. Lastly, even if we assume that the increased leucine concentration caused an increased KIC concentration in the liver of ethanol-treated rats, it still would be negligible in comparison to the KIC concentration (1.0 mmol/L) used for measuring the flux.

Since the liver weight of ethanol-treated rats was significantly greater than that of control rats, we also calculated the flux rate per whole liver. Even with calculation per whole liver, the flux through BCKA dehydrogenase was still significantly ($P < .05$) smaller in ethanol-treated rats than in control rats (225 ± 18 *v* 319 ± 27 μmol/h per whole liver).

Effect of Ethanol Addition

The simplest explanation for our observation is that ethanol or the products of its metabolism inhibited the flux through BCKA dehydrogenase. If this were the case, then addition of ethanol to the perfusion medium should do the same as the chronic feeding of ethanol. To test this

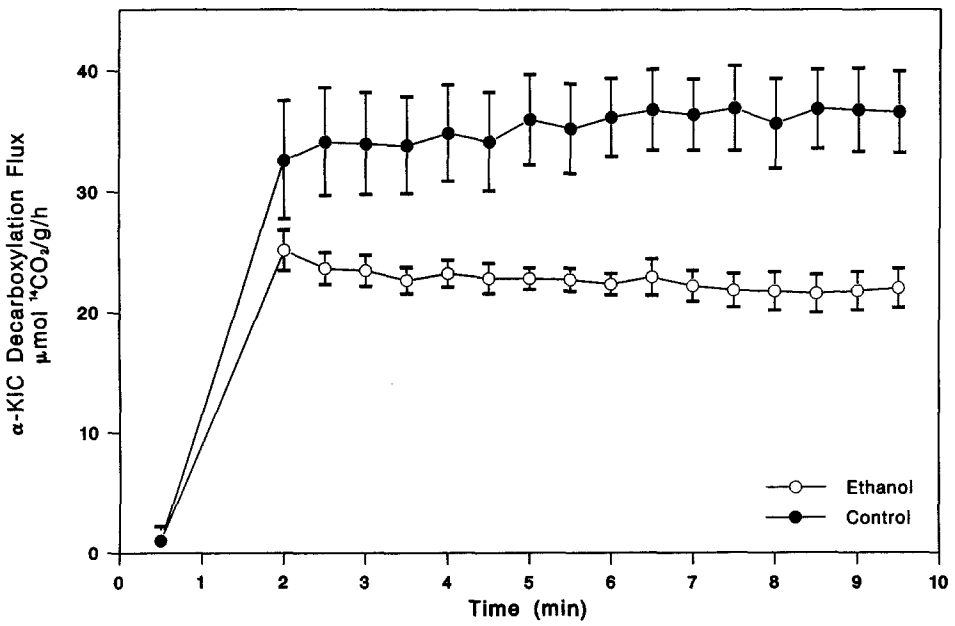

Fig 1. Substrate flux through BCKA dehydrogenase in the liver of rats pair-fed well-balanced liquid diets containing either 0% or 36% of total calories as ethanol for 21 days. After 15 minutes of equilibration, livers were perfused with α-keto[1-^{14}C]isocaproate at a concentration of 1.0 mmol/L for 10 minutes. Mean ± SEM of 6 rats per group.

Table 1. Flux Through BCKA Dehydrogenase in Livers of Control Rats When Perfusion Medium Contained Ethanol

Ethanol (mmol/L)	Flux (μmol/h per whole liver)
0	278 ± 24
25	291 ± 22
50	276 ± 41
100	312 ± 26
200	290

NOTE. After 15 minutes of equilibration, livers of control rats were perfused for 10 minutes with medium containing no ethanol and 1.0 mmol/L α-keto[1-^{14}C]isocaproate. Then, every 10 minutes the perfusion medium was changed to medium containing increasing concentrations of ethanol but the same concentration of KIC. Data are the mean ± SEM for 3 rats, except for 200 mmol/L ethanol, which is for 1 rat.

possibility, livers of control rats were perfused consecutively with media containing 0, 25, 50, 100, and 200 mmol/L ethanol. The concentration of KIC in each medium was 1.0 mmol/L, and the duration of each perfusion was 10 minutes. Ethanol over the entire range of concentrations had no significant effect on the flux through BCKA dehydrogenase in the liver of control rats (Table 1).

Activity State of BCKA Dehydrogenase

Under normal conditions, liver BCKA dehydrogenase is mostly in the active form.[2] Therefore, we hypothesized that chronic ethanol intake decreases the flux by increasing conversion of BCKA dehydrogenase from the active to inactive form. To investigate this hypothesis, we determined basal and total activities (units per gram of liver) of BCKA dehydrogenase in the liver of eight control and eight ethanol-fed rats. Total activity was measured after conversion of all of the enzyme to the active form. Chronic ethanol consumption resulted in significantly ($P < .01$) lower basal and total BCKA dehydrogenase activities (Fig 2) than in control rats. However, ethanol had no significant effect on the activity state of BCKA dehydrogenase in the liver of ethanol-fed and control rats (90% ± 4% *v* 99% ± 3%). The activity state is an index of interconversion of the enzyme between active and inactive forms. Therefore, it appears that chronic ethanol consumption has a catabolic effect on either the synthesis or degradation of BCKA dehydrogenase.

These results regarding the effect of chronic ethanol feeding on the activity of liver BCKA dehydrogenase are at variance with results of a previous study.[10] At least three factors may explain the discrepancy between results of the two studies. First, in the previous study ethanol was administered in the drinking water and not as a component

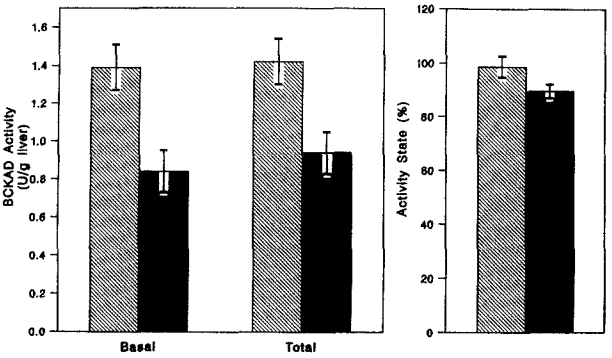

Fig 2. Basal and total BCKA dehydrogenase (BCKAD) activity and activity state in livers of rats pair-fed well-balanced liquid diets containing either 0% (▨) or 36% (■) of total calories as ethanol for 21 days. BCKAD activity was determined spectrophotometrically using 0.5 mmol/L α-keto-isovalerate as a substrate. The enzyme source was a partially purified preparation obtained from nonperfused livers. Differences between control and ethanol-treated rats in basal and total BCKAD activity were statistically significant ($P < .01$), but differences in activity state were nonsignificant. Activity state is the proportion of BCKAD present in the active form. Mean ± SEM of 8 livers per group.

of the diet, to ensure an elevated plasma ethanol level. Second, unlike the present study, no precaution was taken to prevent activation of BCKA dehydrogenase in vitro. Third, the enzyme source in the previous study was not purified enzyme, but a tissue homogenate.

CONCLUSION

The reduction in the flux through liver BCKA dehydrogenase in ethanol-treated rats is inversely proportional to the increase in liver BCAA concentrations produced by chronic ethanol feeding.[1] This finding suggests that decreased oxidation (Fig 1) is one factor that explains the increases in concentrations of BCAA[1] in livers of ethanol-fed rats. In tissues with high BCAA transaminase activity, a high BCAA concentration stimulates its own oxidation by increasing BCKA concentrations, which in turn inhibit BCKA dehydrogenase kinase.[11] However, in the liver, due to the low BCAA transaminase activity, this is not likely to occur.[11] To our knowledge, the present results provide the only known in vivo model for a metabolically induced depression in the activity of liver BCKA dehydrogenase in the face of an increased concentration of BCAA. This model might be of further use for investigating the regulation of BCKA dehydrogenase activity by means other than covalent modification.

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