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Short Communication

CHANGES IN ACTIVITY LEVELS AND ISOZYME PATTERNS OF ISOLEUCINE AMINOTRANSFERASE IN RESPONSE TO EXPERIMENTALLY INDUCED HEPATIC LESION

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Abstract—Branched-chain amino acid aminotransferase activities were measured in rats in which hepatic lesions were induced experimentally, that is, fatty liver produced by an orotic acid-containing diet and acute hepatic lesion induced by D-galactosamine injection into the abdominal cavity. The levels of the enzyme activities, using L-isoleucine as substrate, were elevated in both cases. Among the isozymes (enzymes I, II and III) of the enzyme, the activity of enzyme I was elevated by orotic acid treatment. However, with D-galactosamine treatment, another isoleucine aminotransferase activity was chromatographically separated from that of enzyme I and is considered to be enzyme III, and not enzyme II, based on its substrate specificity.

Key words: orotic acid; D-galactosamine; hepatic lesion; isoleucine aminotransferase; isozyme of isoleucine aminotransferase; methionine adenosyltransferase

The branched-chain amino acid aminotransferase (EC 2.6.1.42) is widely distributed among tissues with high activity in the heart and kidney [1, 2]. The unique feature of branched-chain amino acid aminotransferase in animals is its distribution. Most enzymes involved in amino acid metabolism are located in the liver, but the activity of the branched-chain amino acid aminotransferase is low in the liver.

The isozymes of branched-chain amino acid aminotransferase from various tissues in humans and rats have been separated by DAEA-cellulose column chromatography [3, 4]. In rats, all tissues examined contain enzyme I, whereas enzyme III is predominant in the brain, ovary and placenta, and appears to be expressed in fetal or transformed tissues [4]. L-Valine, L-leucine and L-isoleucine are substrates of both enzymes I and III. Enzyme II, which acts only on L-leucine among the three branched-chain amino acids, has been found only in rodent livers [3, 5]. The strict specificity of enzyme II for L-leucine and its high K_m values suggest that this enzyme may act in the regulation of L-leucine metabolism. This is further supported by the fact that enzyme II is induced markedly by various treatments, such as the administration of a high protein diet or cortisol [6, 7]. However, these treatments do not cause any significant changes in the activity of another isozyme (enzyme I) found in the liver. D-Galactosamine-induced hepatitis in mice causes elevation of the activities of both aspartate aminotransferase and alanine aminotransferase in serum [8]. However, the effect of the treatment on the activities and isozyme forms of branched-chain amino acid aminotransferase in liver is not clear. We report here the changes in activities and isozyme patterns of isoleucine aminotransferase in the livers of rats treated with orotic acid or D-galactosamine.

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Materials and Methods

Male Wistar strain rats, 3 weeks of age, were used in these experiments. Rats for the control group were fed 30 g/day for 3 weeks of a standard diet and were given water *ad lib*. The standard diet consisted of: 24.6 g protein, 5.6 g lipid, 6.4 g ash, 3.1 g fiber, 52.3 g soluble non-nitrogen compounds, 8.0 g water, and 358 kcal as total calories. Male Wistar strain rats were also used for the induction of fatty liver by an orotic acid diet. They were fed 30 g/day for 3 weeks of a diet of the following composition: 1.0 g orotic acid, 73.5 g sucrose, 18.0 g casein, 0.3 g choline, 4.0 g McCollum salt, 2.0 g corn oil and 1.2 g vitamin mixture.

For induction of acute hepatic lesion by D-galactosamine, male Wistar strain rats were fed a standard diet for 3 weeks, and then D-galactosamine solution (0.5 g/mL of 0.9% NaCl) was injected into the abdominal cavity at a dose of 500 mg/kg rat weight. After the rats fasted for 24 hr, the liver was removed, homogenized, centrifuged into mitochondrial and supernatant fractions, and used as the enzyme preparation. The results described here are given as the means of values obtained from 3 to 10 rats. Standard laboratory chow was obtained from the Oriental Yeast Co. Ltd.; other nutritional materials were from commercial sources.

Determination of isoleucine aminotransferase activity. The activity of isoleucine aminotransferase was assayed spectrophotometrically by a slight modification of the method described by Ichihara and Koyama [1]. The standard reaction mixture contained in 1.5 mL: 50 μmol sodium pyrophosphate buffer, pH 8.6, 10 μmol α-ketoglutarate, 0.1 μmol pyridoxal phosphate, 10 μmol L-isoleucine, and the enzyme fraction. The reactions were initiated by the addition of L-isoleucine after preincubation at 37° for 5 min, were carried out for 20 min, and were terminated by the addition of trichloroacetic acid at a final concentration of 5% (w/v). The amount of keto acid formed

Table 1. Effects of orotic acid and D-galactosamine treatments on isoleucine aminotransferase activity in rat liver and heart

Treatment	No. of rats	Enzyme activity (nmol/mg protein/20 min)		Lipid content (%)
		Mitochondria	Supernatant	
Liver				
None	7	10.9 ± 5.0	16.3 ± 6.5	5.6
Orotic acid	5	16.8 ± 4.5	70.7 ± 7.1*	21.6
D-Galactosamine	3	30.3 ± 1.8*	58.2 ± 2.4*	12.3
Heart				
None	7	308.5 ± 47.5	994.7 ± 65.0	
Orotic acid	5	214.5 ± 37.1†	1036.5 ± 170.3	
D-Galactosamine	3	570.4 ± 54.0*	1053.2 ± 70.5	

Experimental details on orotic acid and D-galactosamine treatments and assay conditions are described in Materials and Methods. Rat organs were homogenized with 0.25 M sucrose containing 3 mM Tris–HCl, pH 7.4, and 0.1 mM EDTA. After the homogenates were centrifuged at 700 *g* for 10 min, supernatant fractions were centrifuged at 10,000 *g* for 15 min. Precipitates were resuspended with the homogenized solution and used as the mitochondrial fraction containing the enzyme activity. The supernatant fraction of 10,000 *g* was also used for the enzyme assays. Lipid content is expressed as the ratio to each liver wet weight. Data are the means ± SD from more than three experiments. Statistical analysis was performed using Student's *t*-test.

*,† Significantly different from the control value (none): * $P < 0.001$ and † $P < 0.01$.

was then quantified spectrophotometrically at 440 nm after the formation of its 2,4-dinitrophenylhydrazone, using a modification of the method of Wada and Snell [9]. The 2,4-dinitrophenylhydrazone of α-keto-β-methylvalerate was used as the standard. Activity was expressed as nanomoles of keto acid formed per 20 minutes under the above assay conditions.

Assay of methionine adenosyltransferase (EC 2.5.1.6) activity [10, 11]. The assay medium contained in a final volume of 0.25 mL: 100 mM Tris–HCl, pH 7.8, 200 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM ATP, 5 mM L-methionine, 7.4 kBq of L-[*methyl*-^{3}H]methionine, and enzyme preparation. At the end of each incubation period at 37°, 20 mL of ice-cold water was added to the test tube containing the assay mixture, and the diluted solution was then passed over a column (0.9 × 2.0 cm) of Dowex 50W. Radioactive *S*-adenosylmethionine, in contrast to L-methionine, is retained on Dowex 50W at neutral pH. After complete passage of the solution, the column was washed with 5 mL of water, and subsequently the radioactive product was eluted with two 5-mL aliquots of 3 N NH_4OH. Each eluate was added to 15 mL of Atom Light for scintillation counting. Assay blanks were obtained from an incubation in the absence of enzyme.

Protein concentrations were measured by the method of Bradford [12] using bovine serum albumin as the standard or by the ratio of absorbance at 280 and 260 nm in the case of chromatographic fractions. Lipid content was measured by the modified method of Folch *et al.* [13, 14]. For statistical analysis of the data, Student's *t*-test was used.

Results

The isoleucine aminotransferase activities of the centrifugal fractions prepared from the homogenate of normal rat liver were measured as the control; the activity of the supernatant fraction was higher than that of the mitochondrial fraction. In the orotic acid-treated livers, the activity of the supernatant fraction was elevated dramatically compared with that of the normal rat livers (Table 1). The lipid content of the livers treated with orotic acid increased about 4-fold compared with that of the control rat group. Although the activity of isoleucine aminotransferase from the normal rat heart was much higher than that of the normal rat liver, the activity from the heart supernatant was not elevated by treatment with orotic acid (Table 1).

In rat liver treated with D-galactosamine, the activities of both the supernatant and the mitochondrial fractions were enhanced, and the content of lipid also increased (Table 1). In contrast, the activity of the mitochondrial fraction from rat heart treated with orotic acid decreased, while that of the mitochondrial fraction treated with D-galactosamine increased as was the case for the liver. The activity of the supernatant fraction from rat heart treated with D-galactosamine did not change.

To elucidate the mechanisms of the elevation of activity levels in both supernatant fractions prepared from the fatty liver treated with orotic acid and the liver damaged by D-galactosamine, we carried out an investigation of the isozyme patterns of isoleucine aminotransferase for both supernatant fractions, using column chromatography. It is well known that three types of isozymes (enzymes I, II and III) of branched-chain amino acid aminotransferase occur in rats. The experimental data for the isozyme patterns of the isoleucine aminotransferase from normal, orotic acid-treated, and D-galactosamine-treated rat livers are shown in Fig. 1. As seen in Fig. 1A, the elution pattern from the DEAE-cellulose column of the supernatant fraction from the normal rat liver revealed that enzyme I was the main component among the three isozymes, when L-isoleucine was used as the substrate in the assays. In the fatty liver caused by orotic acid treatment, enzyme I was accompanied by an elevated specific activity (Fig. 1B).

On the other hand, isoleucine aminotransferase activity from liver damaged by D-galactosamine was shown to be separated into two fractions (Fig. 1C). Compared with Fig. 1A, the first active fraction eluted must be enzyme I, whereas the activity in the second fraction eluted with 0.2 to 0.24 M potassium phosphate freshly appeared in the liver treated with D-galactosamine. This second fraction

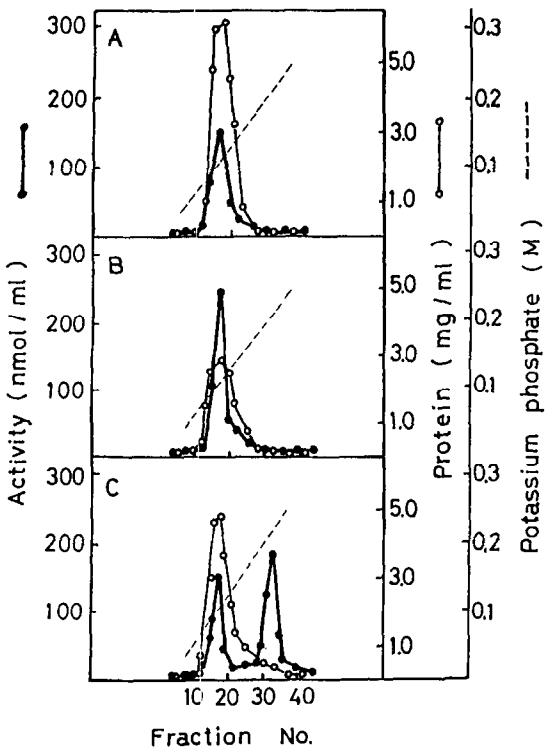

Fig. 1. DEAE-cellulose column chromatography of the supernatant fraction of rat liver treated with orotic acid and D-galactosamine. The supernatant fluids were dialyzed against Buffer I (5 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA and 1 mM 2-mercaptoethanol). The dialyzed preparations were applied to a DEAE-cellulose column (1.0 × 15 cm) equilibrated with Buffer I, and the column was washed with a column volume of Buffer I. The enzyme was then eluted with a linear concentration gradient of 60 mL of Buffer I and 60 mL of Buffer II (0.3 M potassium phosphate, pH 7.8, 0.1 mM EDTA and 1 mM 2-mercaptoethanol). Fractions (3 mL) of eluate were collected. An aliquot (0.9 mL) of each fraction was assayed for isoleucine aminotransferase activity of normal (A), orotic acid-treated (B) and D-galactosamine-treated (C) rat livers. Isoleucine aminotransferase activity is expressed in nanomoles of keto acid produced per milliliter of each fraction. Key: isoleucine aminotransferase activity (●——●); potassium phosphate concentration (---); and protein concentration (○——○).

with high specific activity was considered to be enzyme III, because the activity was obtained when measured with L-isoleucine as the branched-chain amino acid substrate, and L-isoleucine is not a substrate for enzyme II [4]. L-Valine and L-leucine were also used as substrates for the second fraction (data not shown).

The effects of the above treatments with orotic acid or D-galactosamine on methionine adenosyltransferase activity in liver were also measured. The enzyme activity in the supernatant fraction was decreased to less than one-half with orotic acid treatment and to one-third with D-galactosamine compared with that of the normal liver (Table 2). The enzyme activities of the mitochondrial fractions were not changed as much as those of the supernatant fractions.

Discussion

The possibility of a relationship between the elevation of isoleucine aminotransferase activity and lipid content in rat liver after treatment with orotic acid (shown in Table 1) may be expected from the following results. To confirm this phenomenon, the subsequent experiment was carried out. After treatment with orotic acid for 3 weeks, the rats were further fed a standard diet for 3 days. The lipid content of the liver obtained after this treatment decreased to 8.7% from 21.6% for the liver of the rat treated with orotic acid alone, and isoleucine aminotransferase activity of the liver from the rat treated with orotic acid and then standard diet as above also decreased to 2-fold activity from 4.3-fold activity for the liver from the rat treated with only orotic acid, compared with that of the normal rat liver. There was a good parallel relationship between the decrease of the lipid content and that of the isoleucine aminotransferase activity. These results are compatible with the consideration that orotic acid interferes with glycosylation of lipoprotein, thus accumulating very low density lipoprotein (VLDL) in the Golgi apparatus of the liver cell and inhibiting the release of VLDL from the liver to the plasma [15, 16].

Other experimental results obtained by us showed that methionine adenosyltransferase activities from the livers of the orotic acid- and D-galactosamine-treated rats decreased to less than one-half and one-third of that from the normal liver, respectively (Table 2). It is also known that D-galactosamine directly and rapidly disrupts hepatic RNA and protein synthesis [17].

From the D-galactosamine-treated liver, another activity, which was considered to be that of isozyme III of branched-chain amino acid aminotransferase, also appeared on the DEAE-cellulose column chromatogram (Fig. 1). In this connection, hepatoma cells have been found to express the enzyme III gene, and the possibility of alteration of the enzyme III gene expression in hepatoma cells is inferred and discussed [5].

The above observations reported by other researchers

Table 2. Effects of orotic acid and D-galactosamine treatment on methionine adenosyltransferase activity in rat liver

Treatment	No. of rats	Enzyme activity (μmol/mg protein/30 min)	
		Mitochondria	Supernatant
None	7	0.15 ± 0.02	2.52 ± 0.39
Orotic acid	5	0.03 ± 0.058*	1.18 ± 0.04†
D-Galactosamine	3	0.14 ± 0.015	0.80 ± 0.02†

Experimental details on orotic acid and D-galactosamine treatments and assay conditions are described in Materials and Methods. Data are the means ± SD from more than three experiments. Significance was determined by Student's *t*-test.

*,† Significantly different from the control (none): * $P < 0.01$ and † $P < 0.001$.

and our experimental results suggest that some common mechanisms that give rise to alteration of the isozyme gene expression, between the process with the action of D-galactosamine and that with the transformation of liver cells, are functioning.

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