Mechanism of Liver Tyrosine Aminotransferase Increase in Ethanol-Treated Mice and Its Effect on Serum Tyrosine Level

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Summary Liver tyrosine aminotransferase (TAT) activity is known to increase with ethanol treatment; however, the mechanism of this increase is unclear. Upon investigation we found that TAT activity and mRNA levels started to increase 2 h after ethanol administration and continued to increase until 6 h after ethanol administration. The increase in ethanol-induced TAT activity could not be explained by calorie loading after fasting, since ethanol loading increased TAT expression, while glucose loading decreased TAT expression. In addition, liver TAT activity was not related to serum tyrosine levels. TAT activity increased when an adenosine A2 agonist, 5′-N-ethylcarboxamide adenosine, was given. Since TAT activity is increased by cAMP, and ethanol increases cAMP production via an adenosine receptor-dependent mechanism, this increase in ethanol-induced TAT activity may occur via an adenosine receptor-dependent mechanism.

Key Words tyrosine aminotransferase, ethanol, branched-chain amino acid, serum glucose, adenosine A2 receptor

Tyrosine aminotransferase (TAT; EC 2.6.1.5) is mainly found in the liver, where it catalyzes the first reaction in the pathway by which tyrosine is finally degraded to acetoacetate and fumarate (1). TAT is one of the enzymes induced by acute ethanol treatment (2), and at 4 h after ethanol administration, the enzyme activity is unaltered in fed rats, but there is a significant increase in starved rats (2). This ethanol-induced increase in hepatic TAT activity involves acceleration of enzyme synthesis (3), and is not affected by adrenalcotony or by pyrazol-induced inhibition of ethanol metabolism (3). The mechanism by which ethanol increases TAT activity is still unclear.

The Fischer ratio (branched-chain amino acids to aromatic amino acids ratio) and the branched-chain amino acids to tyrosine ratio (BTR) are used as dynamic markers of nutritional assessment in many clinical situations (4–7). Changes in serum tyrosine level, which may be due to several causes, affect both the Fischer ratio and BTR values, which may in turn interfere with the accuracy of clinical assessment. This raises the question whether changes in TAT activity affect the serum tyrosine level, thereby impacting on clinical assessments. Two previous reports that examined serum tyrosine levels found contradictory results, with one reporting that liver TAT was related to the serum tyrosine level, while the other reporting that it was not. In particular, Suzuki et al. suggested that ethanol accelerates the intrahepatic metabolism of tyrosine via an insulin effect, which reduces the tyrosine level (8).

However, Henderson et al. reported that TAT activity in the cirrhotic liver was not significantly different from TAT activity in the noncirrhotic liver; therefore, the elevated tyrosine levels noted in cirrhotics cannot be explained by decreased TAT activity in the liver (9). It is still unclear whether liver TAT activity affects the serum tyrosine level.

In the present study, we investigated the mechanism by which ethanol increases TAT activity and determined whether increased liver TAT activity affects the serum tyrosine level.

MATERIALS AND METHODS

Reagents. All reagents used were of analytical grade and were purchased from Nacalai Tesque Ltd. (Kyoto, Japan) unless otherwise stated. 5′-N-ethylcarboxamide adenosine (NECA) and N^6-phenylisopropyladenosine (PIA) were purchased from Sigma-Aldrich Co. Japan. [α-32P]dCTP was purchased from Amersham Biosciences, USA.

Animal treatments. Male mice (ddY strain, 4-wk-old, 20–25 g) were purchased from Japan SLC and maintained on laboratory food (MF, Oriental Yeast Co., Ltd., Japan) and water provided ad libitum under an 8:00 to 20:00 light/20:00 to 8:00 dark cycle. In the fasting experiments, performed as described previously (10), food was withheld from animals for either 10 h or 16 h, starting at 8:00 (Experiment 1, n = 3 for each time period). The 16 h-fasting experiment was repeated to confirm the change in TAT activity (Experiment 2, n = 6). For the ethanol-treatment experiments, after
fastering, the mice were orally administered ethanol (25% ethanol in saline: 3 mL/100 g body weight) 0, 1, 2, 4 or 6 h prior to liver collection (Experiment 3, n=2), or orally administered ethanol (0, 8, 16, or 25% ethanol in saline: 3 mL/100 g body weight) 6 h prior to liver collection (Experiment 4, n=3). The livers were collected at 16:00 in all mice.

For the calorie-loading experiment, after 16 h of fasting, the mice were orally administered ethanol (25% ethanol in saline: 3 mL/100 g body weight) or orally administered glucose (0.48 g glucose/mL saline: 3 mL/100 g body weight) 6 h prior to liver collection (Experiment 5, n=10).

In the adrenalectomy experiment, mice were adrenalectomized and maintained for 5 d with 0.9% NaCl as drinking water, and after 16 h of fasting, the mice were orally administered ethanol (25% ethanol in saline: 3 mL/100 g body weight) 6 h prior to liver collection (Experiment 6, n=2 or 3).

In the experiment involving adenosine agonists for TAT activity, after 16 h of fasting, the mice were intraperitoneally administered NECA (0.03 mg/mL saline/100 g body weight) or PIA (0.03 mg/mL saline/100 g body weight), and the livers were then collected 6 h after administration (Experiment 7, n=3). To determine the change in mRNA level, experiment 7 was repeated with NECA treatment (Experiment 8, n=6 or 7). The doses used were based on the findings of previous studies (11, 12). In all experiments, the control mice were treated with the same volume of saline. Blood was obtained under ether anesthesia, and the serum was prepared by centrifugation. Tissue samples were collected, immediately frozen in liquid nitrogen, and then stored at -80°C until analysis. All procedures were performed in accordance with the Kobe Gakuin University Guidelines for the Care and Use of Laboratory Animals.

RNA preparation and hybridization analysis. Total RNA was extracted using the acid guanidine thiocyanate-phenol-chloroform method (13). Details of the northern hybridization have been previously described (10). A rat TAT fragment (nucleotides 31 to 862 of cDNA fragment GenBank accession No. Y13346) and the 18S rRNA fragment were labeled using the [α-32P]dCTP and a Random Prime DNA labeling Kit Ver. 2.0 (Takara Shuzo, Japan) and used as probes.

**TAT activity and serum amino acid measurement.** TAT activity was determined according to the alkali-catalyzed conversion method of the reaction product, p-hydroxyphenylpyruvate, to p-hydroxybenzaldehyde (14). For amino acid measurement, serum (0.05 mL) was deproteinized with 0.8 M HClO4 (0.1 mL), and then 0.1 M of the supernatant was neutralized with 4 M KOH (13 μL). After centrifugation, 40 μL of the supernatant was concentrated using a Speed Vacuum Concentrator (Savant, USA) and resuspended in 0.1 M HCl (25 μL). After that, the amino acids were measured using the PTC-amino acid method by HPLC with a Pico-Tag column (15 cm × 3.9 mm, Waters, Japan) (15).

**Other analytical methods.** The protein concentration was measured by the dye-binding method using a protein assay (Bio-Rad, Japan). Bovine serum albumin was used as the standard. Glucose was measured using the Glucose C2 Test Wako (Wako Pure Chemical, Japan).

**Statistical analysis.** All statistical analyses were performed using the statistical software package, Prism ver. 4.0 (GraphPad Software, Inc., USA).

**RESULTS**

**TAT expression in ethanol-treated mouse livers**

Ribiere et al. reported that ethanol only increases TAT activity in the livers of fasting rats (2). To further understand the conditions under which fasting activity in the fasting mouse liver was determined. In normal rats, TAT activity has a circadian variation (16, 17). Therefore, the fasted mouse liver TAT mRNA levels were compared with the levels in fed mice obtained at the same time. As shown in Fig. 1A, though TAT mRNA increased in the 16-h fasted liver, the increase was not

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**Fig. 1.** The effect of fasting on mouse liver TAT expression. (A) The effect of fasting on TAT mRNA expression (Experiment 1). The mean±SE of the ratio of each TAT mRNA to the level of the loading control 18S rRNA signal is shown under the photograph. (B) The effect of fasting on TAT activity (Experiment 2). Each data point is shown as the mean±SE. The livers were collected at 10:00 in 16 h normally fed mice and 16 h fasted mice. The non-paired Student's t-test was used to compare a fasted group with its related fed group.
TAT Increase in Ethanol-Treated Mice

Fig. 2. The effect of ethanol on mouse liver TAT expression. (A) Time-dependent changes in the TAT mRNA level and TAT activity caused by ethanol (Experiment 3). (B) The effects of ethanol concentration on TAT mRNA and TAT activity (Experiment 4). The mean ± SE of the ratio of the TAT mRNA level to the loading control 18S rRNA signal level is shown under the photograph. For TAT activity, each data point is shown as the mean ± SE. The mice were treated with ethanol for 0–6 h after fasting started at 18:00, and the livers were collected at 16:00 of the next day in all mice. ANOVA with Tukey was used to compare the groups. Values without a common superscript letter are significantly different (p<0.05).

Fig. 3. The effect of calorie loading on mouse liver TAT expression (Experiment 5). (A) The effect of calorie loading on TAT mRNA. Each data point is the mean ± SE of the ratio of each TAT mRNA level to the loading control 18S rRNA signal level. (B) The effect of calorie loading on TAT activity. Each data point is shown as the mean ± SE. The mice were treated with each substance after 16 h of fasting, and 6 h after the treatment, the livers were collected at 16:00 in all mice. ANOVA with Tukey was used to compare the groups. Values without a common superscript letter are significantly different (p<0.05).

statistically significant. The TAT activity in the fasted mouse liver after 16 h of fasting was not changed compared with that in fed mouse liver (Experiment 1, data not shown). To confirm the absence of any difference between TAT activity in fasted mice liver and in fed mice liver, the fasting experiment was repeated (Experiment 2). As shown in Fig. 1B, there was no significant difference in TAT activity between fasted and fed mice. Based on this finding ethanol was given orally to fasting mice 0, 1, 2, 4, or 6 h prior to liver collection (Experiment 3). Figure 2A shows the liver TAT levels over the 6 h after ethanol administration. The TAT mRNA level and activity started to increase at 2 h after ethanol administration, continued to increase until 6 h, and then returned to the basal level at 24 h (data not shown). The mice were also treated with several different ethanol concentrations for 6 h (Experiment 4, Fig. 2B). Compared to the 0% ethanol-treated group, only the 25% ethanol-treated group had a significant increase in the mRNA level after 6 h of treatment. Compared to the level of the 0% ethanol group, TAT activity also increased 2.5-fold 6 h after 25% ethanol treatment. The TAT activity of fasting mice (0% ethanol) varied from 1.5 to 40 nmol/min per mg of protein.

Mechanism of ethanol-induced TAT increase

To investigate whether caloric loading influences TAT activity, the mice were given an amount of glucose that was equal in calories to that of the 25% ethanol treat-
Fig. 4. The effect of ethanol on liver TAT expression in adrenalectomized mice (Experiment 6). (A) TAT mRNA. The mean±SE of the ratio of each TAT mRNA level to the loading control 18S rRNA signal level is shown under the photograph. (B) TAT activity. Each data point is shown as the mean±SE. After 16 h of fasting, ethanol or saline was given orally 6 h before tissue collection, and the livers were collected at 16:00 in all mice. C: control mouse, E: ethanol-treated mouse, S: saline treated, sham-operated mouse. The non-paired Student’s t-test was used to compare the 6E group with the 6C group. Values without a common superscript letter are significantly different (p<0.05).

Fig. 5. The effect of NECA on mouse liver TAT expression. (A) The effect of NECA on TAT mRNA (Experiment 8). The mean±SE of the ratio of each TAT mRNA level to the loading control 18S rRNA signal level is shown under the photograph. (B) The effect of NECA on TAT activity (left: Experiment 7; right: Experiment 8). Each data point is shown as the mean±SE. The mice were treated with each substance after 16 h of fasting, and 6 h after NECA or PLA administration, the livers were collected at 16:00 in all mice. ANOVA with Tukey for more than three groups or the non-paired Student’s t-test for two groups was used for statistical analysis. Values without a common superscript letter are significantly different (p<0.05).
NECA had significantly increased TAT activity in the liver, while PIA and adenosine had not affected TAT activity. To determine the effect of NECA on the mRNA level, an additional experiment was performed (Experiment 8). NECA significantly increased TAT mRNA as well as TAT activity (Fig. 5A and B right).

**Serum amino acid and glucose levels**

To determine the effect of TAT activity on serum tyrosine level, and to compare the effects on amino acid metabolism of ethanol, glucose, and NECA treatment, serum amino acid and glucose levels in each experiment were determined. As shown in Table 1, tyrosine levels decreased significantly in the fasting mice (Experiment 2). In the calorie-loading experiment (Experiment 5), there was no difference in the tyrosine levels among the control, ethanol and glucose groups, while TAT activity changed significantly (Fig. 3). The serum tyrosine level did not correlate with liver TAT activity.

The branched chain amino acid level was compared between the fasted mice of Experiment 2 (total fasting time: 16 h) and the control mice of Experiment 5 (total fasting time: 22 h). We found that the 22 h-fasted mice had a higher serum branched chain amino acid level than the 16 h fasted mice. With glucose loading, the branched-chain amino acid level decreased, while it did not decrease in ethanol-loaded mice. In addition, when compared to the control group of Experiment 5, the serum glucose levels of the glucose-loaded group increased. After ethanol loading, the glucose level also increased significantly. With NECA treatment (Experiment 8), both the serum branched-chain amino acid level and the glucose level increased significantly.

**DISCUSSION**

In this paper, two new findings about TAT and liver function were identified. First, that TAT activity increases due to an adenosine agonist, and second, that liver TAT activity does not affect the serum tyrosine level.

The mechanism by which acute ethanol treatment increases TAT was investigated. TAT is an enzyme that is induced by glucocorticoids (20), glucagon (21), and dibutyl cyclic adenosine 3',5'-monophosphate (cAMP) (22), and TAT activity is also increased by acute ethanol treatment in fasting rats (2). Our results (Fig. 2) also show that mouse liver TAT activity increases following ethanol treatment. To determine the mechanism by which ethanol increases TAT, the mice were given an amount of glucose that had an equivalent number of calories to the 25% ethanol treatment. Suzuki et al. noted that a significant elevation in serum immunoreactive insulin levels followed the elevation of serum glucose levels after alcohol loading; they suggested that insulin accelerates the intrahepatic metabolism of tyrosine (8). However, our results showed that opposite changes in TAT occurred after calorie-loading with ethanol compared to glucose, while both ethanol- and glucose-loading increased serum glucose levels (Fig. 3 and Table 1). These findings indicate that the effect of ethanol on TAT expression cannot be explained by an insulin effect. Furthermore, glucocorticoids were not involved in the ethanol-induced increase in TAT mRNA levels (Fig. 4).

Next, to better understand the effects of adenosine on TAT activity, the mice were treated with an adenosine agonist, either NECA or PIA. Nagy reported that acute exposure of primary rat hepatocyte cultures to ethanol increases the extracellular adenosine concentration (19), and that ethanol increases cAMP production in cultured hepatocytes via an adenosine receptor-dependent mechanism (23). Adenosine receptors have been divided into three major subclasses, A1, A2, and A3. The A1 receptor mediates the inhibition of adenylate cyclase; the A2 receptor mediates the stimulation of the enzyme (24) by affecting Gi and Gs proteins, coupled to adenylate cyclase (25); and the A3 receptor, when expressed in Chinese hamster ovary cells, leads to the inhibition of adenylate cyclase, and in a rat basophilic
cell line, the A3 receptor stimulates phospholipase C (26, 27). The adenosine agonist, NECA, has an equal affinity for both A1 and A2 receptors, and a low affinity for A3 receptors, while PIA has a higher affinity for A1 than for the A2 and A3 receptors (27–29). Ethanol increases the extracellular adenosine concentration (19). Our data show that NECA increased TAT activity (Fig. 5), which suggests the possibility that ethanol increases TAT activity via an adenosine A2 receptor-dependent mechanism. Adenosine is rapidly degraded by adenosine deaminase in the blood (30); therefore, adenosine itself did not increase TAT activity (Fig. 5).

The effects of ethanol and NECA on gluconeogenesis were similar (Table 1). In the calorie-loading experiment, the serum alanine level in the control mice (22 h fasted mice) and ethanol-loaded mice was lower than in the glucose-loaded mice. This indicates that gluconeogenesis from alanine was decreased only in the glucose-loaded mice. The serum alanine level in ethanol-loaded mice was lower than in the control mice, but not significantly. The serum glucose level in the ethanol-loaded mice increased significantly compared to the control mice. Ethanol itself cannot be converted to glucose, because ethanol is a ketogenic compound. Based on all these findings it is thought that gluconeogenesis was stimulated in the ethanol-loaded mice. NECA also stimulated gluconeogenesis. The effects of ethanol and NECA on gluconeogenesis were similar.

Glucose given to fasting mice decreased TAT mRNA levels (Fig. 3A). In neonates, which must withstand a brief period of starvation and hypoglycemia, glucocorticoids alone are not sufficient for neonatal liver gene induction; the hypoglycemia that occurs at birth is the main trigger for this expression (31). Feeding causes a rapid decrease in the phosphoenolpyruvate carboxykinase (PEPCK) and TAT mRNA levels. In neonates, both PEPCK and TAT mRNA levels decrease markedly during the first 2 h of refeeding, and then remain almost constant (32). In hepatocytes exposed to insulin for a short period of time, glucocorticoid- and cAMP-induced transcription of the TAT gene is inhibited (33). The results we obtained following glucose treatment after 16 h of fasting were the same as those seen with refeeding after fasting.

In fasting mice who were calorie-loaded, each treatment caused specific changes in the serum amino acid levels (Table 1). Compared to normally fed mice, tyrosine levels decreased in the fasted mice. Currently, the mechanism by which tyrosine decreases is unclear, because TAT activity does not affect the serum tyrosine level (Fig. 3 and Table 1). Compared to the glucose-loaded mice, in the control or ethanol-loaded mice, the serum branched-chain amino acid levels were higher. Most of branched-chain amino acids are not degraded in liver, but rather are exported to and degraded in muscle, for use as an energy source (34, 35). Based on the present results, transport to muscle or the oxidation rate of branched-chain amino acids in muscle may differ between fasted or ethanol-loaded mice, and glucose-loaded mice.

The reason why ethanol increases TAT activity only in fasting mice is still unknown. Further investigation is required to fully understand the effects of ethanol or diet on liver function.

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REFERENCES