Note

Galactosamine-Induced Acute Liver Injury in Rats Reduces Hepatic α -Tocopherol Transfer Protein Production

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Summary The liver plays the main role in the secretion of food-derived α -tocopherol into the circulation through the functioning of α -tocopherol transfer protein (α -TTP). However, the effect of liver disease on α -TTP level and α -tocopherol metabolism has not been clarified. We examined the amount of liver α -TTP and its effect on serum α -tocopherol concentration in liver injury. Male Wistar rats were injected intraperitoneally with D-galactosamine at 800 mg/kg body weight, and liver and serum lipid concentrations, α -tocopherol concentrations, and hepatic α -TTP mRNA and protein levels were measured at 24, 48, and 72 h after injection. On the basis of body weight changes and serum transaminase activities, the livers were found to be in an injured state 24 and 48 h after galactosamine injection but had recovered by 72 h. The hepatic α -TTP mRNA level was reduced throughout the experimental period, and at 48 h after injection the α -TTP protein level had begun to decrease. Lipid and α -tocopherol concentrations in the serum were decreased at 24 and 48 h after injection and increased at 72 h. Liver lipid concentrations were increased at 24 and 48 h after injection, but the liver α -tocopherol concentration was unchanged. These results show that galactosamine-induced liver injury decreases hepatic α -TTP synthesis in rats. Serum α -tocopherol concentration was not directly affected by the acute change in hepatic α -TTP level, suggesting that the chronic changes in α -TTP activity would be necessary to regulate serum α -tocopherol concentration.

Key Words α -tocopherol transfer protein, α -TTP, galactosamine, liver injury, rat

α -Tocopherol is the most active form of vitamin E and is the most potent lipid-soluble antioxidant in biological membranes. After intestinal absorption, vitamin E is packaged into chylomicrons and carried to the liver. α -Tocopherol transfer protein (α -TTP), a liver cytosolic protein, binds to α -tocopherol with higher affinity than to other forms of vitamin E and transfers α -tocopherol to lipoproteins in the circulation (1). It has now been established that α -TTP, through this mechanism, is a major determinant of plasma α -tocopherol level. In fact, humans with a defective α -TTP gene have severe vitamin E deficiency (2), and depletion of the α -TTP gene in mice results in severely reduced plasma and tissue α -tocopherol concentrations (3–5). Although genetic α -TTP deficiency severely affects serum and

peripheral tissue α -tocopherol concentrations, effects of the reduction of hepatic α -TTP level on serum or liver α -tocopherol under physiological conditions have been reported in only a very few cases (6, 7).

Since liver α -TTP plays a major role in supplying α -tocopherol to the circulation, reduced α -tocopherol secretion into the blood might occur in animals with liver injury. In fact, decreased serum α -tocopherol concentrations have been reported in patients with hepatitis (8–10), although the effects of various liver diseases on serum α -tocopherol are still controversial (11). This study was designed to determine the effects of liver injury on hepatic α -TTP level and liver and serum concentrations of α -tocopherol, with galactosamine-induced liver injury as an animal model of human hepatitis.

Galactosamine-induced liver injury is a commonly used animal model of human hepatitis. After injection, galactosamine is metabolized to UDP-hexosamines and UDP-N-acetylhexosamines in the hepatocytes, transiently blocks transcription and protein synthesis in those cells, and induces necrosis in the hepatic parenchymal cells (12). Recent studies have demonstrated that apoptosis also contributes to galactosamine-induced liver injury by increasing TNF- α production

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Abbreviations: AGPC, acid guanidium thiocyanate-phenol-chloroform; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; PL, phospholipids; TG, triglyceride; α -TTP, α -tocopherol transfer protein; TC, total cholesterol.

(13–15).

We injected male Wistar rats intraperitoneally with D-galactosamine, and measured their liver and serum α -tocopherol concentrations and hepatic α -TTP mRNA and protein levels at 24, 48, and 72 h after injection.

Materials and Methods

Materials. Anti-rat α -TTP monoclonal antibody was prepared by Sato et al. as described previously (16). Other chemicals were of reagent grade, available commercially.

Animal experiment. The care and use of the rats followed the institutional guidelines of Yamagata University. Six-week-old male Wistar rats (Japan SLC, Hamamatsu, Japan) were housed individually in stainless-steel cages with a 12 h light:dark cycle (06:00–18:00) at a temperature of 22–24°C and a relative humidity of about 55%. The rats were allowed free access to water and fed a commercial diet for 3 d to allow acclimation to these conditions.

The rats were divided randomly into six groups ($n=5$ for control groups and $n=8$ for galactosamine-injected groups), and rats in all groups were fed normal diets consisting of 200 g/kg casein, 50 g/kg corn oil, 437 g/kg α -cornstarch, 218 g/kg sucrose, 50 g/kg cellulose, 10 g/kg vitamin mixture (AIN97, obtained from Oriental Yeast Co., Tokyo, Japan), and 35 g/kg mineral mixture (AIN97, obtained from Oriental Yeast Co.). Water and food were provided ad libitum. On day 4, three groups of rats were injected intraperitoneally with D-galactosamine dissolved in water at a dose of 800 mg/kg body weight, and rats in the other groups were injected with water. Rats from each group were anesthetized and blood was collected by cardiac puncture at 24, 48, or 72 h after galactosamine injection. A section of the liver was excised, quickly frozen in liquid nitrogen, and stored at -80°C until use.

Determination of liver and serum α -tocopherol concentrations. α -Tocopherol in the liver and serum was extracted according to the procedure of Harada et al. (17). α -Tocopherol concentration was determined fluorometrically by high-performance liquid chromatography as described previously (18).

Determination of serum ALT, AST, and LDH activities. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined with the GPT-UV test Wako and GOT-UV test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively. Lactate dehydrogenase (LDH) activity was measured as described by Wroblewski and LaDue (19). Briefly, serum (50 μL) was added to 2 mL of 50 mmol/L phosphate buffer (pH 7.5) containing 0.62 mmol/L lithium pyruvate and 0.18 mmol/L NADH, which had been prewarmed at 35°C . The rate of decrease of absorbance at a wavelength of 340 nm was measured. LDH activity (unit/mL) was defined as 1 unit = $1,720 \times (\text{decrease in absorbance at 340 nm for 2 min})$.

Analysis of lipid content in serum and liver. Liver lipids were extracted and purified by the method of Folch et al. (20). The triglyceride (TG), phospholipid (PL) and total

cholesterol (TC) contents of the serum and liver were determined with Triglyceride E-test kit, Phospholipid B-test kit and Cholesterol E-test kit, respectively (Wako Pure Chemical Industries, Ltd.).

Northern blot analysis. Total RNA was prepared from the liver according to a modified method of acid guanidium thiocyanate–phenol–chloroform (AGPC) extraction (21, 22), as described previously (23). Northern blot analysis was performed as described previously (23). The cDNA fragment of rat α -TTP (517–792 bp) was obtained by RT-PCR and was used as a probe. The intensity of each band obtained from Northern blot analysis was quantified by the Fujix BAS 1800 system (Fuji Film Co., Tokyo, Japan).

Western blot analysis. A sample of 0.2 g of liver was homogenized on ice in buffer (50 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 1% (w/v) Triton X-100, 0.1% SDS, 0.1 mg/mL phenylmethanesulfonyl fluoride, 0.05 mg/mL aprotinin, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin). The homogenized liver was centrifuged (20,100 $\times g$, 4°C , 20 min) twice, and the supernatant of the second centrifugation was used as the liver protein sample. Ten micrograms of each protein sample was electrophoresed and transferred to a nylon membrane (Hybond-N+, Amersham Japan, Tokyo, Japan). After the membrane had been blocked with BSA and incubated with anti- α -TTP antibody (1 : 1,000) followed by probing for 1 h with anti-mouse IgG conjugated with horseradish peroxidase (1 : 1,000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), α -TTP was detected by measuring chemical luminescence using ECL Western Blotting kit (Amersham Japan) and a lumino-image analyzer LAS1000 system (Fujix, Tokyo, Japan).

Statistics. Data are expressed as means $\pm$ SE. The homogeneity of variance between treatments was verified by Bartlett's test. Differences between two groups were tested by Student's *t*-test. Differences were considered significant at $p < 0.05$ (Statcel, OMS Publishing Inc., Saitama, Japan).

Results and Discussion

To elucidate whether galactosamine injection induced liver injury, we measured food intake, body weight, and serum transaminase activity. The food intakes of galactosamine-injected rats were reduced to 75 to 80% of the control level. Body weight gains were also reduced until 48 h after galactosamine injection and then increased between 48 and 72 h after injection. The liver weight of galactosamine-injected rats was reduced 48 h after injection but had recovered by 72 h (Table 1). Serum activities of aminotransferases (ALT and AST) and LDH were significantly higher in galactosamine-injected rats than in controls at 24 and 48 h after injection but returned close to control levels by 72 h after injection (Table 1). Galactosamine-injected rats that showed no changes in serum AST, ALT, or LDH activities were regarded as not being liver-injured and samples from those rats were not used in further experiments. These data show that liver injury was present in galactosamine-injected rats 24 and 48 h

Table 1. Effect of β -D-galactosamine injection on food intake, body weight gain, liver weight, serum AST, ALT, LDH activities, and serum and liver lipids concentrations in rats.

	24 h		48 h		72 h	
	con (n=5)	gal (n=5)	con (n=5)	gal (n=6)	con (n=5)	gal (n=7)
Food intake (g)	13.6 $\pm$ 0.2	10.2 $\pm$ 0.3*	27.4 $\pm$ 0.7	20.8 $\pm$ 1.1*	39.9 $\pm$ 0.9	31.3 $\pm$ 1.4*
Body weight gain (g)	5.36 $\pm$ 0.52	-1.42 $\pm$ 0.60*	10.7 $\pm$ 0.5	-0.05 $\pm$ 1.30*	13.4 $\pm$ 0.5	6.41 $\pm$ 0.54*
Liver weight (g)	5.37 $\pm$ 0.05	4.69 $\pm$ 0.09*	5.60 $\pm$ 0.06	4.45 $\pm$ 0.24*	5.39 $\pm$ 0.19	5.56 $\pm$ 0.18
Liver weight (% of body weight)	4.49 $\pm$ 0.05	4.09 $\pm$ 0.08*	4.48 $\pm$ 0.04	3.95 $\pm$ 0.20*	4.22 $\pm$ 0.10	4.63 $\pm$ 0.10*
Serum AST activity (IU/L)	33.4 $\pm$ 3.6	246 $\pm$ 37*	52.2 $\pm$ 5.7	211 $\pm$ 17*	45.9 $\pm$ 6.7	63.0 $\pm$ 3.9*
Serum ALT activity (IU/L)	55.6 $\pm$ 4.8	198 $\pm$ 26*	62.5 $\pm$ 4.5	180 $\pm$ 12*	74.5 $\pm$ 4.9	73.5 $\pm$ 4.9
Serum LDH activity (IU/L)	387 $\pm$ 64	1319 $\pm$ 131*	483 $\pm$ 38	628 $\pm$ 34*	540 $\pm$ 57	470 $\pm$ 73
Serum TG (mg/mL)	0.891 $\pm$ 0.044	0.359 $\pm$ 0.040*	0.992 $\pm$ 0.136	0.681 $\pm$ 0.071	0.756 $\pm$ 0.084	1.48 $\pm$ 0.07*
Serum PL (mg/mL)	1.48 $\pm$ 0.04	0.741 $\pm$ 0.055*	1.54 $\pm$ 0.02	1.08 $\pm$ 0.11*	1.46 $\pm$ 0.07	2.52 $\pm$ 0.12*
Serum TC (mg/mL)	0.716 $\pm$ 0.021	0.301 $\pm$ 0.029*	0.841 $\pm$ 0.024	0.627 $\pm$ 0.072*	0.748 $\pm$ 0.030	1.49 $\pm$ 0.06*
Liver TG (mg/g liver)	4.38 $\pm$ 0.41	13.3 $\pm$ 1.5*	5.02 $\pm$ 0.52	10.1 $\pm$ 1.5*	5.63 $\pm$ 0.64	6.73 $\pm$ 0.44
Liver PL (mg/g liver)	15.0 $\pm$ 0.9	17.4 $\pm$ 0.4*	14.5 $\pm$ 0.4	14.6 $\pm$ 0.3	15.8 $\pm$ 0.3	14.1 $\pm$ 0.2*
Liver TC (mg/g liver)	1.76 $\pm$ 0.11	2.87 $\pm$ 0.08*	1.84 $\pm$ 0.05	3.17 $\pm$ 0.25*	1.99 $\pm$ 0.06	2.25 $\pm$ 0.08*

AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; TG, triglyceride; PL, phospholipid; TC, total cholesterol; con, control group; gal, β -D-galactosamine-injected group.

Values are expressed as means $\pm$ SE.

* Significant differences compared with control group.

Fig. 1. Hepatic α -TTP mRNA and protein level in control rats (con) and β -D-galactosamine-injected rats (gal) at 24, 48, and 72 h after injection. (A) A representative result of Northern blot analysis is shown together with levels of membrane-transferred 28S rRNA. (B) The intensity of each band in Northern blotting was determined and expressed as a value relative to that of the controls. (C) A representative result of Western blot analysis is shown. (D) The intensity of each band in Western blotting was determined and expressed as a value relative to that of the controls. Values are means $\pm$ SE and are expressed relative to those of the control group. *Significantly different from the control group, $p < 0.05$.

after injection, but that the galactosamine-injected rats had recovered from the injury by 72 h after injection; these observations are consistent with those of a previous report (24).

Because α -tocopherol is lipid soluble and is transported in lipoproteins, liver and serum lipid concentrations were measured to allow comparisons with α -tocopherol concentration. Serum TG, PL, and TC concentrations

of galactosamine-injected rats were lower than those of controls 24 and 48 h after injection, and significantly higher 72 h after injection. Liver TG and TC concentrations were significantly higher in galactosamine-injected rats than in controls 24 and 48 h after injection, but by 72 h they had returned close to those in the controls. Liver PL concentration was higher in galactosamine-injected rats than in controls 24 h after injection.

Fig. 2. Liver and serum α -tocopherol concentrations in control rats (solid lines) and β -D-galactosamine-injected rats (dashed lines) at 24, 48, and 72 h after galactosamine injection. Values are means $\pm$ SE. *Significantly different from control group, $p < 0.05$.

tion but was lower at 72 h than in controls (Table 1). Galactosamine-injected rats therefore had fatty livers 24 and 48 h after injection, as described previously, probably because of decreased β -oxidation (25).

α -TTP mRNA and protein contents were measured to elucidate the effect of galactosamine-induced acute liver injury on α -TTP synthesis. Typical results of Northern blot analysis, together with the amount of 28S rRNA transferred to the nylon membrane, are shown in Fig. 1A. The hepatic α -TTP mRNA level was significantly lower in galactosamine-injected rats 24 and 48 h (20–30% of the control level), and 72 h (50% of the control level) after galactosamine injection (Fig. 1B), indicating that the α -TTP mRNA level was influenced directly by the severity of liver injury. The reduced expression of hepatic α -TTP mRNA was probably caused by the induction of necrosis and apoptosis in the liver parenchymal cells or by the reduction of liver-specific function by the galactosamine injection (26, 27). Although the food intake of the galactosamine-injected rats was reduced throughout the experimental period, this reduction was not the main cause of the decline in α -TTP mRNA levels, because restriction of food intake (48-h fasting) did not affect hepatic α -TTP mRNA level in our previous study (data not shown).

The α -TTP protein content had begun to decrease in galactosamine-injected rats by 48 h after galactosamine injection and at 72 h was still lower than that in the controls (30% of the control level) (Fig. 1C, D). The relative amount of α -TTP in whole liver was also significantly lower in galactosamine-injected rats at 48 and 72 h after injection (1.02 ± 0.05 at 24 h, 0.57 ± 0.05 at 48 h and 0.52 ± 0.07 at 72 h, relative to a control group value of 1). The time lag between the decreases in α -TTP mRNA and protein levels suggested that α -TTP protein had a long half-life and was therefore not influ-

enced immediately by decreased mRNA expression.

To examine the effect of hepatic α -TTP content on α -tocopherol metabolism, liver and serum α -tocopherol concentrations were measured. Although it has been reported that hepatic α -tocopherol concentration is well correlated with liver lipid concentrations in patients with liver disease (11) and in galactosamine-injected rats (28), we found that hepatic α -tocopherol concentration was unchanged and was not correlated with liver lipid concentrations (Fig. 2A, B). These results suggest that hepatic lipid and α -tocopherol concentrations are regulated independently. Serum α -tocopherol concentration was lower in galactosamine-injected rats 24 h after injection and higher 72 h after injection than in controls (Fig. 2C). Therefore, despite the decreased hepatic α -TTP protein level in galactosamine-injected rats at 48 and 72 h after injection, the serum α -tocopherol concentration did not differ from that in control rats 48 h after injection and was even higher 72 h after injection. These results showed that a reduction in hepatic α -TTP level did not immediately reduce serum α -tocopherol concentration. The amount of α -TTP in the liver may be excess to transfer α -tocopherol from the liver to the blood, or the α -tocopherol pool in the serum and peripheral tissues may have been too large to be affected by the acute reduction in α -tocopherol secretion by the liver. On the other hand, the serum α -tocopherol concentration was well correlated with serum lipid concentrations (Fig. 2D). The tocopherol-to-lipid ratio in the serum remains unchanged in liver cirrhosis patients despite a low serum α -tocopherol level (29), and the serum α -tocopherol concentration is well correlated with the serum cholesterol concentration in patients with liver diseases (8). These results indicate that lipids and α -tocopherol might have a common machinery—such as an ATP-binding cassette transporter or

microsomal transfer protein—by which they are secreted from the liver. α -TTP may function to transport α -tocopherol to these transport molecules. Taken together, the available evidence indicates that serum α -tocopherol concentration is correlated with serum lipid concentration but is not affected by hepatic α -TTP level in animals with acute liver injury.

Since α -TTP knockout mice have very low α -tocopherol concentrations in the serum and peripheral tissues, it has been suggested that extremely low levels of hepatic α -TTP could reduce α -tocopherol transfer activity from the liver to the serum (3–5). In the case of α -TTP hetero-knockout mice, in which hepatic α -TTP content is about 50% of that in normal animals, serum and peripheral α -tocopherol concentrations at 3 mo of age are 30% lower than normal (4). Therefore, a 50% decrease in α -TTP in this study should have been sufficient to affect the serum α -tocopherol concentration. Probably, a decrease in α -TTP production over a longer period would have reduced the serum α -tocopherol concentration.

The decreased serum α -tocopherol concentrations seen at 24 and 48 h after injection may have been induced by the accelerated consumption of α -tocopherol under increased oxidative stress. It has been reported that galactosamine injection increases lipid peroxidation and decreases the contents of non-enzymatic antioxidants (glutathione, vitamin C and vitamin E) in rats; these alterations were normalized by oral intake of an antioxidant (18, 30). However, it is not easy to estimate the contribution of α -tocopherol consumption in decreasing serum α -tocopherol level in galactosamine-injected animals, because antioxidant treatment may inhibit liver injury and maintain serum α -tocopherol levels as well as reduce α -tocopherol consumption. As the changes in serum α -tocopherol concentration were well correlated with lipid concentrations in our study, liver injury may affect serum α -tocopherol level mainly through the alteration of lipid transport.

In conclusion, these results indicate that galactosamine-induced liver injury decreases α -TTP production in rats; however, serum α -tocopherol concentration is not directly affected by the acute changes in hepatic α -TTP level.

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