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ORIGINAL ARTICLE

Mechanism of the suppression against D-galactosamine-induced hepatic injury by dietary amino acids in rats

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Abstract To elucidate the mechanism by which dietary amino acids suppress the D-galactosamine (D-GalN)-induced hepatitis, we examined the involvement of Kupffer cells, tumor necrosis factor-α (TNF-α) and apoptosis in the mechanism. In experiment 1, the rats were fed with 10% L-glutamine or 5% glycine diet injected with D-GalN with or without gadolinium chloride ($GdCl_3$)-pretreatment. The results indicated that these amino acids suppressed the D-GalN-induced elevation of serum transaminase activities, irrespective of $GdCl_3$-pretreatment. In experiment 2, rats were fed with 10% of L-glutamine, L-serine, L-alanine or L-glutamic acid diets injected with D-GalN. The results demonstrated that all these amino acids suppressed the D-GalN-induced elevation of serum transaminase activities, but that serum TNF-α concentrations and hepatic caspase-3 activities in the rats were not appreciably changed. In conclusion, the suppressive effects of amino acids on D-GalN-induced hepatitis were suggested not to be always mediated by the inhibition of Kupffer cells → TNF-α → apoptosis pathway.

Keywords L-Alanine · Gadolinium chloride · D-Galactosamine · L-Glutamic acid · L-Glutamine · L-Serine

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Abbreviations

$GdCl_3$	Gadolinium chloride
D-GalN	D-Galactosamine
Enzymes:AST	Aspartate aminotransferase, EC 2.6.1.1
ALT	Alanine aminotransferase, EC 2.6.1.2
TNF-α	Tumor necrosis factor-α

Introduction

D-galactosamine (D-GalN) causes hepatic injury in experimental animals and its histopathological characteristics are similar to human viral acute hepatitis in experimental animals (Keppler et al. 1968). D-GalN was suggested to induce hepatic injury associated with the by-decrease of UTP concentration in hepatocyte, resulting in inhibition of mRNA and protein synthesis (Decker and Keppler 1972). On the other hand, previous researches demonstrated that bacterial translocation caused by the increase of gut permeability (Galanos et al. 1979) and resulted endotoxemia were induced by D-GalN treatment (Kasravi 1996). Bacterial translocation was shown to be blocked by gut excision or treatment with antibiotic which could reduce bacteria in the animal gut (Mihas et al. 1990). Since Kupffer cells are well known to be activated by the endotoxin derived from intestine after D-GalN injection (Galanos et al. 1979), D-GalN-induced hepatitis has been suggested to be initialized by apoptosis which is caused by inflammatory cytokines such as, tumor necrosis factor-α (TNF-α) released from Kupffer cells and then to be resulted in the hepatocyte death by necrosis (Stachlewitz et al. 1999).

A critical degree of liver cell death due to apoptosis or necrosis is considered to be fundamental for the development of fulminant hepatic failure (Riordan and Williams 2003),

and an activation of the pro-inflammatory cytokine cascade including TNF-α is considered to play an important role in the pathophysiology and clinical outcome of this severe liver injury (Romics et al. 2004). Kupffer cells are reported to be increased in number and considered closely related to the upregulation of cytokines and chemokines in fulminant hepatic failure (Leifeld et al. 2003). In our previous study, the diets containing wheat gluten, which is rich in L-glutamine residue which was shown to suppress the induction of hepatitis caused by D-GalN (Katayama et al. 1996; Manabe et al. 1996). Moreover, similar suppressive effects of other amino acids such as, L-asparagine, L-serine and L-histidine as well as glycine and L-glutamine were observed in the D-GalN induced liver injury (Wang et al. 1999). However, the mechanism of such suppressive effects has not been elucidated yet. Concerning the mechanism of glycine-mediated suppression, the action was estimated to be caused by the activation of a chloride channel on the Kupffer cell membrane, and resulted in enhancement of Cl^--influx and hyperpolarization of the cell membrane that would prevent opening of voltage-dependent calcium channels on the plasma membrane and blunt increase in intracellular calcium (Stachlewitz et al. 1999).

In the present study, we examined whether Kupffer cells would be involved in the mechanism of suppressive effects of several amino acids on the D-GalN-induced hepatitis by using gadolinium chloride in experiment 1, and then investigated the action of the amino acids in relation to apoptosis at early and late stage of hepatitis by measuring mediators such as, serum TNF-α concentration and hepatic caspase-3 activity in the next experiment.

Materials and methods

Chemicals

The following materials were obtained commercially: D-galactosamine hydrochloride (D-GalN) and gadolinium chloride hexahydrate ($GdCl_3$) from Wako Pure Chemical Industries, Ltd (Japan). Glycine, L-glutamine, L-serine, L-alanine, L-glutamic acid were provided by Kyowa Hakko Kogyo Co., Ltd (Japan). A kit for transaminase assay, Iatrozyme TA-LQ was purchased from Mitsubishi Kagaku Iatron (Japan). Rat TNF-α assay kit, Rat TNF-α Quantitative colorimetric kit was obtained from Biosource International (USA). Hepatic caspase-3 was analyzed with a CPP32/caspase-3 fluorometric protease assay kit (Medical and Biological Laboratories, Japan).

Animal experimental designs

Male Wistar rats aged 4–5 weeks were obtained from CLEA Japan (Japan), and were kept in an environmentally controlled room at a temperature of 22 ± 1°C on a 12 h light, 12 h dark cycle (light from 7:00 to 19:00). All the rats were fed with a commercial diet CE-2 (CLEA Japan, Japan) for 2 days and then one of the standard purified diets ST-76 or ST-93 in Tables 1, and 2 for 7 days. Thereafter, the rats were divided into several groups as described below to make the averages and standard deviations of the body weight. These groups were approximately equal, and fed with the experimental diets indicated for individual groups in Table 1. In the experimental diets, the amount of amino acids added was compensatory subtracted from that of cornstarch (for experiment 1) or casein (for experiment 2). Rats were allowed to access freely to the diets and water except for the periods before and after the D-GalN administration as described below in each experimental procedure. Care and treatment of the rats were carried out according to the guidelines prescribed by the Faculty of Horticulture of Chiba University, and National Institutes of Health Guide for the Care and Use of Laboratory animals (National Research Council 1996).

Experiment 1: the comparison of the influence of dietary amino acid diets on the serum transaminase activities in the rats treated with or without $GdCl_3$ after D-GalN administration

After feeding AIN-76 standard diet (ST-76, Table 1, The American Institute of Nutrition 1977) for 7 days as describe above, 36 rats aged 5 weeks were assigned to eight groups. They were fed ST-76 diet (NOR, NOR + Gd, CON, and CON + Gd groups)and 10% L-glutamine (10% GLN, 10% GLN + Gd) or 5% glycine (5% GLY, 5% GLY + Gd) for 8 days, and half groups of the individual diet groups (NOR + Gd, CON + Gd, 10% GLN + Gd

Table 1 Composition of the diets (%) for experiment 1

	ST-76 (for NOR and CON groups)	10% GLN	5% GLY
Casein	20.0	20.0	20.0
L-Glutamine	–	10.0	–
Glycine	–	–	5.0
Gelatinized cornstarch	40.0	30.0	35.0
Sucrose	25.0	25.0	25.0
Corn oil	5.0	5.0	5.0
Vitamine mxture[a]	1.0	1.0	1.0
Mineral mixture[a]	3.5	3.5	3.5
Cellulose	5.0	5.0	5.0
Choline bitartrate	0.2	0.2	0.2
DL-Methionine	0.3	0.3	0.3

[a] Composition of the AIN-76 diet (The American Institute of Nutrition 1977)

and 5% GLY + Gd groups) were treated with $GdCl_3$ solution at the 6th day as described below. The rats in 6 groups (CON, CON + Gd, 10% GLN, 10% GLN + Gd and 5% GLY, 5% GLY + Gd groups) were administered D-GalN solution intraperitoneally on day 7 (24 h after $GdCl_3$ treatment) of the experimental diets. The remaining rats in NOR and NOR + Gd groups were injected with saline solution in the same manner. All of them were allowed to be fasted for 4 h before and 4 h after D-GalN or saline administration (total of 8 h). Blood samples were collected from inferior vena cava of all animals under pentobarbital anaesthesia and livers were excised after 24 h of D-GalN administration.

Experiment 2: effect of dietary amino acids on hepatic caspase-3 activity of the liver and serum TNF-α concentration in the rats after 8 and 24 h of D-GalN injection

One hundred and twenty-eight rats aged 4 weeks were assigned to 16 groups of eight rats each were given AIN-93^G standard diet (ST-93, Table 2, Reeves et al. 1993) for 7 days as described above. Then, for the successive 8 days, one of these groups was given ST-93 (NOR groups), and other groups were fed high protein control (CON groups), 10% L-glutamine (10% GLN groups), 10% L-serine (10% SER groups), 10% L-alanine (10% ALA groups) or 10% L-glutamic acid (10% GLU groups) containing diet, where every 3 groups were given the same diet to examine the effect at 0, 8, 24 h after D-GalN injection. The composition of the diets used in this experiment is shown in Table 3 and all rats were allowed to be fasted for 4 h before and after the D-GalN treatment. The rats in the groups other than ST-93 (NOR group) were injected with D-GalN in the same manner in experiment 1, and all rats were allowed to be fasted for 4 h before and after the D-GalN treatment as described in experiment 1. The blood collection and dissection were operated 0 (immediately before), and 8 and 24 h after D-GalN treatment.

Biochemical analysis

Serum aspartate aminotransferase (AST, EC.2.6.1.1) and alanine aminotransferase (ALT, EC.2.6.1.2) in all experimental animals were measured with commercial assay kit Iatrozyme TA-LQ. For TNF-α concentration, rat serum was diluted twofold with saline and its TNF-α concentration was analyzed with Rat TNF-α Quantitative colorimetric kit(Biosource International, USA). For caspase-3 activities, the liver was homogenized with nine volume of cold 0.25 M sucrose. After centrifugation at $105{,}000 \times g$ for 60 min at 4°C, the supernatant was collected. Caspase-3 activity in the supernatant cytoplasm was analyzed with a CPP32/caspase-3 fluorometric protease assay kit (Medical & Biological Laboratories, Japan).

Preparation and administration of D-GalN and $GdCl_3$ solution

D-GalN hydrochloride was dissolved in distilled water and neutralized by NaOH solution to pH 7.0. After its concentration was adjusted to 300 mg/ml, the solution was sterilized by membrane filtration (0.22 μm). This solution was administered intraperitoneally at the rate of 800 mg/kg body weight. For the preparation of $GdCl_3$ solution, $GdCl_3$ $6H_2O$ was dissolved in saline to make its concentration

Table 2 Composition of the diets (%) for experiment 2

	ST-93 (for NOR group)	CON	10% GLN	10% SER	10% ALA	10% GLU
L-Glutamine	–	–	10.0	–	–	–
L-Serine	–	–	–	10.0	–	–
L-Alanine	–	–	–	–	10.0	–
L-Glutamic acid	–	–	–	–	–	10.0
Cornstarch	39.7486	29.7486	29.7486	29.7486	29.7486	29.7486
Casein	20.0	30.0	20.0	20.0	20.0	20.0
Sucrose	23.2	23.2	23.2	23.2	23.2	23.2
Soybean oil	7.0	7.0	7.0	7.0	7.0	7.0
Cellulose	5.0	5.0	5.0	5.0	5.0	5.0
Vitamine mixture[a]	3.5	3.5	3.5	3.5	3.5	3.5
Mineral mixture[a]	1.0	1.0	1.0	1.0	1.0	1.0
L-Cystine	0.3	0.3	0.3	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25	0.25	0.25	0.25
Tert-butylhydroquinone	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014

[a] Composition of the AIN-93^G diet (Reeves et al. 1993)

Table 3 The effect of D-GalN and gadolinium chloride on the body weight gain, feed intake, liver weight and serum transaminase activities in rats fed 5% glycine- and 10% L-glutamine-containing diets in experiment 1

	NOR*	NOR + Gd*	CON	CON + Gd	10% GLN	10% GLN + Gd	5% GLY	5% GLY + Gd
Initial body weight (g)	157.3 ± 3.8	155.3 ± 9.2	154.0 ± 4.6	156.4 ± 3.92	156.2 ± 3.04	156.8 ± 3.48	156.0 ± 3.6	156.6 ± 4.47
Final body weight (g)	210.0 ± 4.5	214.3 ± 3.7	205.8 ± 3.4	205.8 ± 3.2	208.0 ± 3.2	200.8 ± 4.0	219.6 ± 17.1	202.8 ± 5.8
Weight gain (g/7 days)	52.7 ± 1.0	59.0 ± 6.7	51.8 ± 4.6	49.4 ± 4.9	51.8 ± 5.0	44.0 ± 2.6	43.6 ± 3.7	46.2 ± 2.5
Feed efficiency[1] (%)	43.0 ± 1.2	45.0 ± 3.4	41.4 ± 3.4	40.6 ± 3.2	38.8 ± 2.4	36.6 ± 1.8	38.8 ± 3.1	40.6 ± 1.1
Liver weight (g)	13.4 ± 0.6^{c}	14.3 ± 1.1^{bc}	10.2 ± 0.7^{e}	11.3 ± 0.7^{d}	16.6 ± 0.6^{a}	13.1 ± 0.4^{c}	14.6 ± 0.3^{b}	14.9 ± 0.8^{b}
Ratio[2] (%)	8.2 ± 0.1^{d}	8.4 ± 0.7^{d}	6.5 ± 0.4^{f}	7.1 ± 0.4^{e}	10.5 ± 0.3^{a}	8.4 ± 0.3^{d}	9.3 ± 0.1^{c}	9.4 ± 0.3^{b}

The groups other than NOR and NOR + Gd groups were treated with D-GalN. The groups denoted with "+ Gd" were treated with $GdCl_3$ at 24 h before D-GalN injection. NOR and CON groups were fed with ST-76 diet, and 10% L-glutamine and 5% glycine groups with 10% GLN and 5% GLY diets shown in Table 1, respectively. The details of the procedures are described in materials and methods. Values are mean ± SEM ($n = 5$, $n = 3$*). Values without a common superscript letter are significantly different by Duncan's multiple comparison test ($p < 0.05$)

[1] (Weight gain/Feed intake) × 100, [2](Liver weight/Carcass weight) × 100

20 mg/ml and sterilized as described above for the preparation of D-GalN solution. This solution was administered intravenously at the rate of 20 mg/kg body weight through tail vein.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Duncan's multiple-range test was applied when significant differences were obtained by one-way analysis of variance. The level of significance was $p < 0.05$.

Results

The comparison of the influence of dietary amino acids and $GdCl_3$ on the serum transaminase activities in the rats treated with or without $GdCl_3$ after D-GalN administration

The influence of dietary amino acids and $GdCl_3$ on the D-GalN-induced hepatitis was examined in experiment 1 for that the composition of the experimental diets was based on AIN-76 to enable the results to compare with previous related papers.

Initial and final body weight, weight gain, feed efficiency, liver weight and the ratio of liver weight against carcass weight were presented in Table 3. Initial body weight, final body weight, weight gain had not been significantly different among all groups. Although the feed efficiency in 10% GLN and 5% GLY groups was slightly lower than that of CON group, no significant difference was observed among the groups.

The liver weight and its ratio to carcass weight in CON group, which was administrated with D-GalN, were significantly lower than those in NOR group respectively, indicating the reduction of the liver weight by D-GalN treatment. The NOR + $GdCl_3$ group was almost same as that of NOR group. On the contrary, CON + $GdCl_3$ group was significantly higher than that of CON group. Therefore, $GdCl_3$ was suggested to suppress the shrinkage of liver caused by D-GalN. In the 10% GLN and 5% GLY groups, the values of liver weight were higher than that of CON group and addition of $GdCl_3$ to these groups did not result in the fortification of the suppressive effect on the shrinkage of liver by D-GalN. The values of liver weight ratio [(liver weight/carcass weight) × 100] were shown to have almost the same tendency as those of liver weight in relation to the effects of $GdCl_3$ and amino acids. The activities of serum ALT and AST in experiment 1 were presented in Fig. 1. The activities of serum ALT and AST in CON group were extremely higher than those on NOR group, respectively. The activities of serum ALT and AST in CON + Gd group were approximately 50 and 30% of CON group respectively, indicating that the increase in the activities by D-GalN was suppressed by preceding $GdCl_3$ treatment. On the other hand, the activities of serum ALT and AST in 10% GLN and 5% GLY groups were approximately 10% of those in CON group, demonstrating that the increase in the activities by D-GalN suppressed strongly by ingesting the diets containing these amino acids and that this suppression was stronger than that by $GdCl_3$. Since, the activities in these serum transaminases in 10% GLN + Gd and 5% GLY + Gd groups were not significantly different from those in 10% GLN and 5% GLY groups respectively, suppressive effects of the amino acids and $GdCl_3$ were not considered to be additive.

Table 4 Effects of the amino acid containing diets on the final body weight, body weight gain, food consumption, feed efficiency, eviscerated carcass weight and liver weight of the rats at time 0 h of D-GalN treatment (immediately before D-GalN injection) in experiment 2

	Finalbody weight (g)	Body weight gain (g/day)	Food consumption (g/day)	Feed efficiency[1]	Eviscerated carcass weight (%)[2]	Liver weight (%)[3]
NOR	180.00 ± 2.71^{a}	7.22 ± 0.19^{a}	16.97 ± 0.30^{a}	0.49 ± 0.01^{ab}	73.34 ± 0.25^{c}	7.68 ± 0.14^{a}
CON	172.63 ± 3.36^{a}	7.18 ± 0.21^{a}	13.84 ± 0.29^{bc}	0.52 ± 0.01^{a}	74.97 ± 0.28^{a}	6.17 ± 0.10^{d}
10% GLN	171.43 ± 1.77^{a}	6.62 ± 0.14^{ab}	14.26 ± 0.73^{b}	0.48 ± 0.03^{ab}	73.24 ± 0.42^{c}	7.53 ± 0.24^{ab}
10% SER	160.50 ± 2.72^{b}	5.39 ± 0.22^{c}	12.89 ± 0.29^{c}	0.42 ± 0.01^{b}	73.70 ± 0.33^{bc}	7.61 ± 0.21^{ab}
10% ALA	160.13 ± 4.36^{b}	5.42 ± 0.81^{c}	13.16 ± 0.35^{bc}	0.40 ± 0.06^{b}	74.49 ± 0.57^{ab}	7.12 ± 0.14^{bc}
10% GLU	161.88 ± 1.88^{b}	5.61 ± 0.30^{bc}	13.63 ± 0.29^{bc}	0.41 ± 0.03^{b}	73.99 ± 0.22^{abc}	6.81 ± 0.17^{c}

The groups other than NOR were treated with D-GalN. NOR, CON, 10% GLN, 10% SER, 10% GLU and 10% ALA groups were fed the experimental diets shown in Table 2, respectively. The details of the experimental procedures are described in materials and methods. Values are mean ± SEM ($n = 8$). Values without a common superscript letter are significantly different by Duncan's multiple comparison test ($p < 0.05$)

[1] (Weight gain/Feed intake), [2](Eviscerated carcass weight/Final body weight) × 100, [3](Liver weight/Carcass weight) × 100

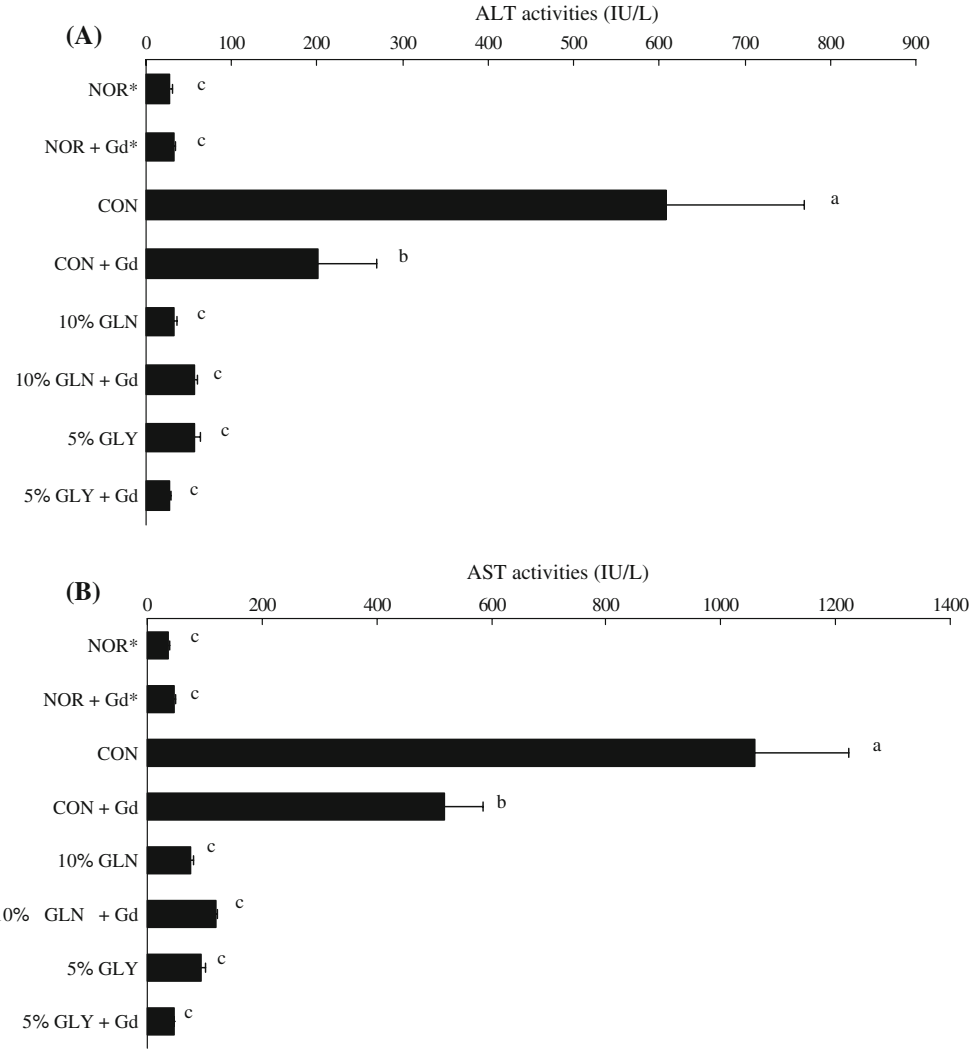

Fig. 1 Effects of high glycine- or high L-glutamine-diet and/or $GdCl_3$ on the serum ALT (A) and AST (B) activities after D-GalN injection. Values are mean ± SEM ($n = 5$, $n = 3$*). Means without a common letter differ significantly ($p < 0.05$). The groups other than NOR and NOR + Gd groups were treated with D-GalN. The groups denoted with "+Gd" were treated with $GdCl_3$ at 24 h before D-GalN injection. NOR and CON groups were fed with ST-76 diet, and 10% GLN and 5% GLY groups with 10% L-glutamine- and 5% glycine-diets shown in Table 1, respectively. The details of the procedures are described in materials and methods

Effect of dietary amino acids on the time dependent alteration of serum transaminase and LDH activities, TNF-α concentration and hepatic caspase-3 activity in the rats after D-GalN-treatment

Experiment 2 was performed to explore the mechanism of the suppressive effect of L-glutamine together with those of L-serine, L-glutamic acid and L-alanine. In this experiment, the compositions of the experimental diets were changed to be based on that for AIN-93^{G}, and the concentrations of protein + amino acids in the diets for groups except NOR group were adjusted to 30.3% to compare the results of the groups precisely. The presentation of the results obtained at 0, 8 and 24 h after D-GalN injection was distinguished by subscript Fig. 0, 8 and 24 after the group names.

The results of growth, food efficiency and the ratio of liver weight against eviscerated carcass weight at 0 h of D-GalN injection (immediately before its injection) in experiment 2 are shown in Table 4. Food consumptions of NOR_0 group, that was fed with ST-93 containing 20.3% (protein + amino acid), was higher than those of other groups feeding the diets containing 30.3% (protein + amino acid), and the body weight gain of 10% SER_0, 10% ALA_0, and 10% GLU_0 groups were slightly but significantly lower than those of NOR_0 and CON_0 groups, indicating that high protein (including amino acids) diets slightly deteriorate their feed intake and growth. Liver weights of the four amino acid containing diet groups were slightly but significantly higher than that of CON_0 group. These results suggest that the supplementation of single amino acid in diet causes high ratio of liver weight to carcass weight. These ratio of liver weight to carcass weight of NOR_{24}, CON_{24}, 10% GLN_{24}, 10% SER_{24}, 10% ALA_{24}, and 10% GLU_{24} (24 h after D-GalN treatment) were 7.68 ± 0.14, 5.25 ± 0.07, 7.74 ± 0.33, 8.12 ± 0.30, 7.68 ± 0.14, 6.12 ± 0.23 and 5.67 ± 0.16, respectively. So, the tendency of elevation of relative liver weight by L-glutamine and L-serine were observed even after D-GalN treatment.

Activities of serum ALT or AST of all groups at 0 h after D-GalN treatment were almost the same (Fig.2). At 8 h after the treatment, ALT and AST activities of CON_8 were two or threefold higher than those of CON_0 at 0 h ($p < 0.05$). However, the elevation of the activities in 10% SER_8 groups was negligible and those in 10% ALA_0, and 10% GLU_0 were smaller than that in CON_0 group. At 24 h after D-GalN treatment, ALT and AST activities of CON_{24} were approximately twentyfold higher than those of CON_0 group ($p < 0.05$). The elevation of transaminase activities in 10% GLN_{24}, 10% SER_{24} were less than twofold of the values of CON_0, and those in 10% ALA_{24}, and 10% GLU_{24} were 4 ∼10-folds higher than CON_0 group. These results indicated that ingestion of L-glutamine or L-serine strongly suppressed the elevation of the serum transaminase activities after D-GalN administration and that the suppressive effects of L-alanine and L-glutamic acid were moderate than that of former two amino acids. The values of serum LDH activity of the groups after D-GalN administration have the similar tendency to those in the case of the serum transaminase activities (data was not shown).

The concentrations of serum TNF-α after D-GalN treatment were shown in Table 5. In the CON_0 group, the TNF-α concentration was elevated approximately 50% at 8 h ($p < 0.05$) and the level was maintained until 24 h after D-GalN treatment. On the other hand, in the group ingested any one of the 4 amino acids, serum TNF-α concentration at 8 h or 24 h after D-GalN injection was not significantly higher than the individual values at 0 h, respectively, and only at 8 h, the concentration of CON_8 group was significantly higher than those of four amino acid diet groups. The concentration of serum TNF-α of 10% GLN_0 and 10% ALA_0 were found to be rather higher than those in 10% SER_0 and 10% GLU_0 groups. These results indicate that the alteration of transaminase activities by these amino acids was not always consistent with the change of their serum TNF-α concentration.

The activities of hepatic caspase-3 are shown in Table 6. The activities of all groups at 0 h after D-GalN treatment were almost the same, indicating that ingestion of the diets containing any one of the four kinds of amino acids or more casein did not affect the activity. In CON groups, the activity of hepatic caspase-3 was elevated only at 24 h after D-GalN injection. Although the caspase-3 activities of 10% ALA_8 and 10% GLU_8 were slightly higher than those of CON_8, 10% GLN_8 and 10% SER_8, those at 24 h after D-GalN treatment were not significantly different among all the groups, indicating that hepatic caspase-3 activities is not related with the amino acid-induced change of the elevation of serum transaminase activity by D-GalN.

Discussion

The present study clearly showed the suppressive effects of dietary glycine, L-glutamine, L-serine, L-glutamic acid and L-alanine on D-GalN-induced hepatic injury. The result of experiment 1 indicated that the elevation of serum ALT and AST activities in rats at 24 h after D-GalN treatment was suppressed by preceding $GdCl_3$-treatment as well as by ingesting 10% L-glutamine- or 5% glycine- containing diets. In this experiment, the concentration of glycine in the diet was set at 5% to avoid toxic effect of excess amount of the amino acid. The suppressive effect of dietary L-glutamine on the induction of hepatitis by D-GalN injection was first reported by Katayama and Manabe (Katayama et al. 1996; Manabe et al. 1996), and then the same suppressive

effects of the diets contained with 5% glycine, 10% L-glutamine, 10% L-serine, 10% L-glutamic acid and 10% L-alanine were reported, in that the degree of suppressive intensity was as follows: 10% L-serine > 5% glycine > 10% L-glutamine > 10% L-glutamic acid > 10% L-alanine (Wang et al. 1999). On the other hand, it was reported that preceding $GdCl_3$-treatment or ingestion of 5% glycine diet inhibited the increase of serum ALT and AST activities in the rats after D-GalN injection (Stachlewitz et al. 1999). Although the amount of $GdCl_3$ and the concentration of glycine for the diet used in the study was the same as those in the present study, injected amount of D-GalN in the latter investigation was more than twice of that in the former case. For this reason, $GdCl_3$ suppressed approximately 50% of the increase of serum transaminase activities by D-GalN in the present experiment in contrast to about 70% in the former report. The suppressive effect of dietary glycine was observed in the present experiment, being consistent with the former report. In the former report, glycine was considered to activate chloride channel and blunt the elevation of Ca^{2+} influx of Kupffer cells and resulted in the reduction of TNF-α excretion from the cells. In addition, glycine was shown to reduce the translocation in endotoxin of bacteria into blood stream from gut. In the present study, L-glutamine was also found to suppress the elevation of serum transaminase activities after D-GalN administration to the same level as in the case with glycine. Although the suppressive effect of L-glutamine on the excretion of TNF-α has not been reported, this amino acid was shown to suppress the translocation of endotoxin from gut (Souba 1990). Therefore, at least a part of the suppressive action of this amino acid on the elevation of the serum transaminase activities may be considered to be caused by this action. Since the amount of $GdCl_3$ used in the present experiment was estimated to be enough to reduce the macrophage activity to less than 20% in vivo (Tiegs et al. 1989), the suppressive actions of these two amino acids, which was stronger than that by $GdCl_3$, may be caused partly by the mechanism other than the Kupffer cell-mediated pathway. The treatment with 5% glycine- or 10% glutamine-diets together with $GdCl_3$ administration did not result in the additive alleviative action on the

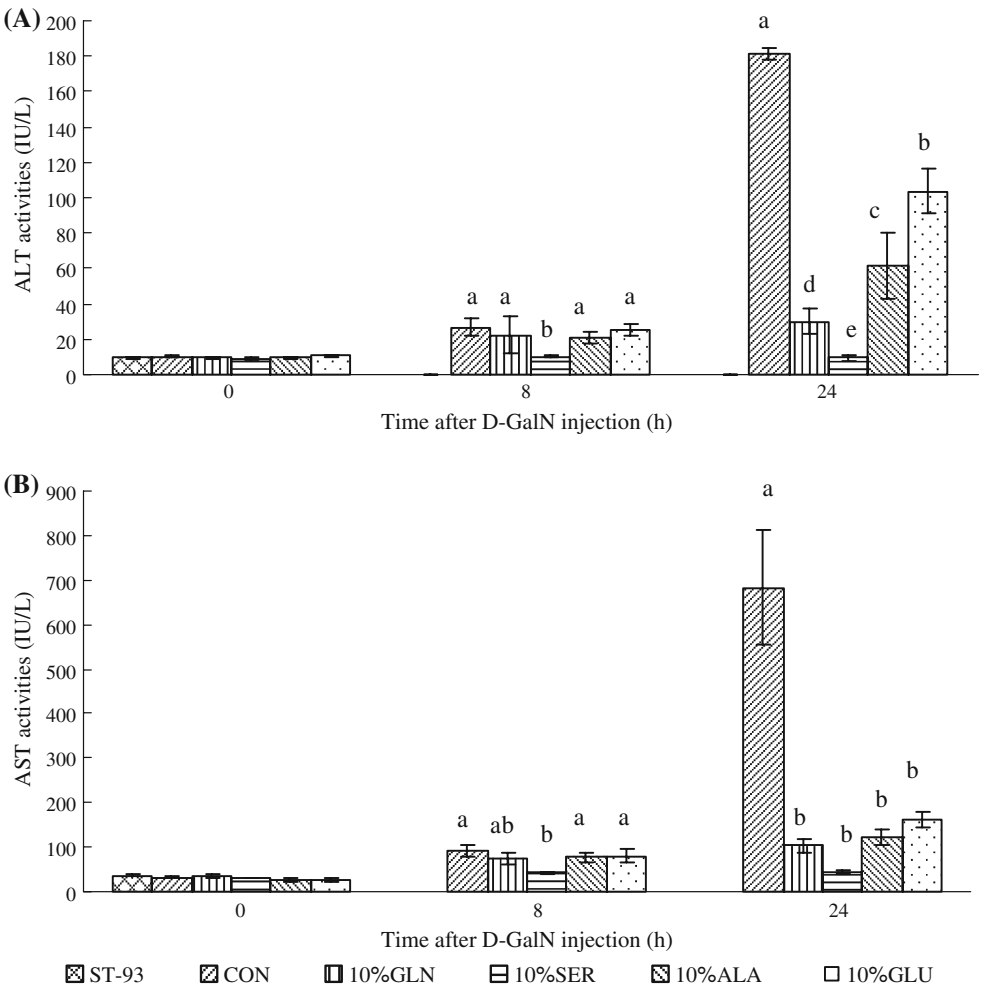

Fig. 2 Effect of D-GalN injection (800 mg/kg) on serum **a** ALT and **b** AST activities of the rats fed with the amino acid-containing diets. Values are means ± SEM ($n = 8$). Means without a comon letter are significantly different at the same time after D-GalN injection ($p < 0.05$). The groups other than NOR were treated with D-GalN. NOR, CON, 10% GLN, 10% SER, 10% GLU and 10% ALA groups were fed the experimental diets shown in Table 2, respectively. The details of the experimental procedures are described in "Materials and methods"

Table 5 Effect of D-GalN injection (800 mg/kg) on serum TNF-α concentrations of the rats fed with the amino acid-containing diets in experiment 2

	Time after D-GalN injection (h)			ANOVA
	0	8	24	
NOR (pg/ml)	8.00 ± 1.34^{bc}	–	–	–
CON (pg/ml)	8.94 ± 1.97^{bc}	13.78 ± 1.08^{a}	11.72 ± 3.04^{a}	$p < 0.05$
10% GLN (pg/ml)	12.73 ± 2.25^{a}	7.60 ± 2.91^{b}	10.30 ± 2.08^{ab}	$p < 0.05$
10% SER (pg/ml)	7.20 ± 2.30^{b}	9.79 ± 1.31^{b}	6.87 ± 1.96^{b}	NS
10% ALA (pg/ml)	10.34 ± 1.60^{ac}	7.64 ± 1.96^{b}	10.30 ± 2.05^{ab}	NS
10% GLU (pg/ml)	7.36 ± 1.39^{b}	9.95 ± 1.00^{b}	7.26 ± 2.27^{b}	NS

The groups other than NOR were treated with D-GalN. NOR, CON, 10%GLN, 10%SER, 10%GLU and 10%ALA groups were fed the experimental diets shown in Table 2, respectively. The details of the experimental procedures are described in materials and methods. Values are mean ± SEM ($n = 5$). Values in a column without a common superscript letter are significantly different at the same time after D-GalN injection by Duncan's multiple comparison test ($p < 0.05$). Time dependent difference in the same dietary group was analyzed by ANOVA. NS: not significant

elevation of serum transaminase activities after D-GalN injection, and the transaminase activities of these groups were almost the same levels as those in 5% glycine- or 10% glutamine-diet group.

In experiment 2, we investigated the effects of four kinds of amino acids, L-glutamine, L-serine, L-glutamic acid and L-alanine in diets on the TNF-α levels and hepatic caspase-3 activities at 8 and 24 h after D-GalN injection. The serum ALT and AST activities in CON group at 8 h after D-GalN injection were slightly but significantly higher than the values at 0 h (Fig. 2). Since previous report indicated that serum ALT and AST activities began to increase around 6 h after D-GalN injection in rats (Manabe et al. 1996), the state at 8 h in the present experiment was thought to be early stage of the hepatic injury. At this stage, only transaminase activities in 10% SER group were significantly lower than those of CON group, respectively. However, the activities in 10% GLN, 10% SER, 10% GLU and 10% ALA groups at 24 h, the later stage, were significantly lower than that in CON group, and the order of suppressive intensity was L-serine > L-glutamine > L-glutamic acid > L-alanine. This order is the same as that indicated in the previous report described above, in that the AIN-76-based diets were used (Wang et al. 1999).

Injection of D-GalN into CON group slightly increased the serum TNF-α concentration at 8 h and this level was maintained till 24 h (Table 5). On the contrary, the concentrations in the 10% SER, 10% GLU and 10% Ala groups were almost unchanged for 24 h, and that in 10% GLN group was rather slightly lower at 8 and 24 h than that at 0 h. Thus, serum TNF-α concentration of all amino acid-ingested groups were slightly but significantly lower than that of CON group at 8 h. These results suggested that TNF-α may possibly be involved in the action only at an early stage of the hepatitis.

In experiment 2, injection of D-GalN increased the hepatic caspase-3 activity in CON group only slightly at 24 h (Table 6). Although apoptosis was estimated to be

Table 6 Effect of D-GalN injection (800 mg/kg) on hepatic cytoplasm caspase-3 activities of the rats fed with the amino acid-containing diets in experiment 2

	Time after D-GalN injection (h)			ANOVA
	0	8	24	
NOR (pmol/mg protein/min)	36.48 ± 11.70	–	–	–
CON (pmol/mg protein/min)	30.60 ± 6.60	31.20 ± 5.49^{b}	55.38 ± 7.45	$p < 0.05$
10% GLN (pmol/mg protein/min)	47.13 ± 13.73	26.22 ± 3.77^{b}	55.03 ± 6.57	NS
10% SER (pmol/mg protein/min)	34.22 ± 3.90	29.34 ± 4.97^{b}	42.06 ± 3.31	NS
10% ALA (pmol/mg protein/min)	45.04 ± 5.15	64.57 ± 11.08^{a}	50.57 ± 13.09	NS
10% GLU (pmol/mg protein/min)	48.22 ± 4.68	57.15 ± 12.79^{a}	68.85 ± 13.40	NS

The groups other than NOR were treated with D-GalN. NOR, CON, 10%GLN, 10%SER, 10%GLU and 10%ALA groups were fed the experimental diets shown in Table 2, respectively. The details of the experimental procedures are described in materials and methods. Values are mean ± SEM, ($n = 5$). Values in a column without a common superscript letter are significantly different at the same time after D-GalN injection by Duncan's multiple comparison test ($p < 0.05$). Time dependent difference in the same dietary group was analyzed by ANOVA. NS: not significant

involved in the D-GalN-induced liver injury (Nan et al. 2004), in the present study the activity of any one of the amino acid-ingested groups did not change significantly for 24 h after D-GalN injection. These results indicate that hepatic activity of caspase-3, an executioner of apoptosis, is not related to the amino acids-dependent suppression of the elevation of serum transaminase activity by D-GalN.

The present study suggested that the suppressive effects of L-serine, L-glutamic acid and L-alanine on the elevation of serum transaminases after D-GalN administration were observed in addition to the similar effects of glycine and L-glutamine. Until now, it has not been investigated that the actions of former 3 amino acids were related with the activities of Kupffer cells, TNF-α and apoptosis of hepatocytes. The results of the present study indicated that all five amino acids used in these experiments were effective and four amino acids other than glycine were found to have suppressive action on the D-GalN-induced hepatitis in spite of the absence of their clear relation with serum TNF-α concentration or hepatic caspase-3 activity. Thus, such function of these amino acids is considered to be caused ubiquitously by the mechanism other than Kupffer cell-mediated event. Because L-Gln is used for nucleotide synthesis as a substrate and the injection of uridine just after D-GalN treatment suppressed the D-GalN-hepatic injury (Keppler et al. 1970), any change in RNA metabolism caused by the ingestion of these amino acids might be involved in such mechanism, although preceding ingestion of uridine-containing diets failed to suppress the D-GalN-hepatic injury (Katayama et al. 1996).

In conclusion, the suppressive effect of dietary amino acids on the D-GalN-induced hepatitis was suggested not to be always mediated by the inhibition of Kupffer cell, TNF-α and apoptosis.

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