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The Antioxidant Effect of DL-α-Lipoic Acid on Copper-Induced Acute Hepatitis in Long-Evans Cinnamon (LEC) Rats

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The Long-Evans Cinnamon (LEC) rats, due to a genetic defect, accumulate excess copper (Cu) in the liver in a manner similar to patients with Wilson's disease and spontaneously develop acute hepatitis with severe jaundice. In this study we examined the protective effect of DL-α-Lipoic acid (LA) against acute hepatitis in LEC rats. LA was administered to LEC rats by gavage in doses of 10, 30 and 100 mg/kg five times per week, starting at 8-weeks-old and continuing till 12-weeks-old. Although LA had little effect against the increases in serum transaminase activities, it suppressed the loss of body weight and prevented severe jaundice in a dose-dependent manner. Antioxidant system analyses in liver showed that LA treatment significantly suppressed the inactivations of catalase and glutathione peroxidase, and the induction of heme oxygenase-1, an enzyme which is inducible under oxidative stress. Furthermore, LA showed dose-dependent suppressive effect against increase in nonheme iron contents of both cytosolic and crude mitochondrial fractions in a dose-dependent manner. Although at the highest dose, LA slightly suppressed the accumulation of Cu in crude mitochondrial fraction, it had no effect on the accumulation of Cu in cytosolic fraction. While LA completely suppressed the increase in lipid peroxidation (LPO) in the microsomal fraction at the highest dose, the suppressive effect against LPO in crude mitochondrial fractions was slight. From these results, it is concluded that LA has antioxidant effects at the molecular level against the development of Cu-induced hepatitis in LEC rats. Moreover, mitochondrial oxidative damage might be involved in the development of acute hepatitis in LEC rats.

Keywords: LEC rats, DL-α-lipoic acid (LA), Copper (Cu), Hepatitis, Reactive oxygen species (ROSs)

Abbreviations: LA, DL-α-lipoic acid; LEC, Long-Evans Cinnamon; Cu, Copper; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; CYT, cytosolic; CMT, crude mitochondrial; LPO, lipid peroxidation; HO-1, heme oxygenase-1; WD, Wilson's disease; ROS, reactive oxygen species; DHLA, dihydrolipoic acid; TP, total protein; GSSG-R, glutathione reductase; MS, microsomal

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INTODUCTION

The LEC rat has been established from a closed colony of Long-Evans rats. The rats usually develop acute hepatitis about 3–4 months after birth, progress to chronic hepatitis, and later develop hepatic cancer from one year after birth.[1,2] LEC rats accumulate excess Cu in the liver[3] but have decreased levels of serum ceruloplasmin activities,[4] a clinical presentation similar to human WD. These animals have a mutation in ATP7B that encodes a P-type adenosine triphosphatase (ATPase), a gene homologous to the WD gene.[5] In both LEC rats and WD patients, absence or dysfunction of the P-type ATPase results in impairment of Cu incorporation into newly synthesized ceruloplasmin in hepatocytes.[5,6] Excessive Cu is implicated as a cause of hepatitis by the accumulation of hepatic Cu found prior to the development of hepatitis and the ability to prevent hepatitis by treatment with Cu-chelating agents such as D-penicillamine.[7] The role of Cu in initiating reactive oxygen species (ROSs) generation[8,9] and consequent oxidative hepatic injury[10,11] has been proposed. Therefore, ROSs generated as a consequence of the accumulation of excess Cu are thought to be responsible for acute hepatitis and hepatic cancer in LEC rats. This mechanism is supported by the fact that a spin-trapping agent, α-phenyl-*tert*-butylnitrone, shows protective effects against hepatitis.[12]

LA plays an essential role in mitochondrial dehydrogenase reactions as lipoamide.[13] LA and its reduced form, dihydrolipoic acid (DHLA), have recently been shown to act as antioxidants *in vitro* and *in vivo*.[14] *In vitro* experiments have shown that LA and/or DHLA reacts with ROSs such as superoxide radicals,[15] hydroxyl radicals,[16] hypochlorous acid,[17] peroxyl radicals,[18] and singlet oxygen.[19] In addition to the ROSs scavenging actions described above, LA and/or DHLA chelate transition metals such as iron[20]and Cu,[21] which are potent catalysts of the Haber-Weiss reaction. LA administration has been shown to be effective in preventing pathology in various experimental models in which ROSs have been implicated, for example, ischemia-reperfusion injury, cataract formation, and diabetes.[14]

A study on the absorption and metabolism of LA has shown that this agent is rapidly absorbed and converted to DHLA[22] in many tissues when administered orally to rats. In the present study, we administered LA orally five times per week to 8-week-old LEC rats for about 4 weeks to evaluate the protective and antioxidant effects of LA against Cu-induced acute hepatitis in LEC rats.

MATERIALS AND METHODS

Animals

Female LEC rats and Wistar rats were purchased from Charles River Japan (Kanagawa Japan) at the age of 7 weeks. The animals were housed in stainless steel cages (3–4 rats/cage) with sterile sawdust for bedding, and maintained in a specific-pathogen free (SPF) facility with the temperature and humidity kept at 23±1°C and 55±5%, respectively. The facility was air-conditioned (10 times per hour) and lighted from 7:00 to 19:00. The rats were acclimatized for 1 week before the experiment and had free access to a standard commercial laboratory chow (F-2, Funabashi Farm, Chiba, Japan) before and during the experimental period. All animals were cared for and treated humanely during the experiment in accordance with our laboratory animal care guidelines.

Administration of LA to LEC rats

DL-α-lipoic acid (LA: Wako Pure Chemical Industries, Ltd., Osaka, Japan) was suspended in 0.5% carboxymethyl cellulose sodium salt (0.5% CMC). The LEC rats were divided into four groups of 7 rats. LA was administered by gavage

five times per week in doses of 10, 30 and 100 mg/kg. Control rats were given 0.5% CMC alone. This was started when the rats were 8 weeks old and continued until they were 12 weeks old (for 4 weeks), when jaundice, a sign of hepatitis, was observed in the control group. Body weight and food intake were measured before every treatment. The Wistar rats were maintained without any treatment until they reached the age of 12 weeks. The rats were euthanized by exanguination from the abdominal aorta under ether anesthesia, and liver and serum samples were taken for the following examinations.

Serum Biochemistry and Hemolysis

Serum biochemical analysis was performed based on measurements of aspartate aminotransferase (AST) activities, alanine aminotransferase (ALT) activities, and concentrations of total bilirubin (T-Bil) and total protein (TP) by an automatic analyzer 7250 (Hitachi, Ltd., Tokyo, Japan). As an index of hemolysis, we measured serum oxyhemoglobin concentration spectrophotometrically.[23] Oxyhemoglobin gives a characteristic peak at 576 nm. Therefore, we defined the difference in optical densities between 576 and 650 nm, OD (576–650) nm, as an index of hemolysis (hemolytic index). Briefly, a 100 μL portion of serum was diluted with 300 μL of phosphate buffered saline (PBS), and this diluent was mixed with a 800 μL solution of 0.5% (v/v) NH_4OH. OD (576–650) nm was recorded against a blank (serum from Wistar rat) on a U-3300 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). Hemolytic index was expressed as OD (576–650) nm.

Preparation of Hepatic Subcellular Fractions

Livers were homogenized in 3 volumes of ice-cold 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer containing 1.15% KCl (pH 7.4). The homogenate was then centrifuged for 10 min at 700g. The resulting supernatant was designated as 700g supernatant. The remaining supernatant obtained was centrifuged for 20 min at 9000g. The resultant pellet was resuspended in 10 mM Hepes buffer (pH 7.4) and designated as the crude mitochondrial fraction (CMT). The remaining supernatant was centrifuged for 60 min at 105,000g and the supernatant used as cytosolic fraction (CYT). The pellet was resuspended and centrifuged for 60 min at 105000g once more, and then resuspended in 10 mM HEPES buffer (pH 7.4). This fraction was designated as microsomal fraction (MS). Each fraction was stored at −80°C until use. The protein contents of all fractions were determined using the Bio-Rad DC protein assay kit (Bio-Rad, Cambridge, MA, USA).

Biochemical Assays

Superoxide dismutase (SOD) activities were determined using an SOD-525 Assay kit (Oxis International Inc., Portland, OR, USA). Glutathione peroxidase (GPX) activity was determined as described by Paglia *et al.* using H_2O_2 as a substrate.[24] Catalase (CAT) activity was measured as described by Beers and Sizer.[25] Glutathione reductase (GSSG-R) activity was assayed according to the method of Carlberg *et al.*[26] Cu content was determined spectrophotometrically as described by Kadiiska *et al.*[27] The standard curve was obtained using $CuSO_4$ as a standard. Non-heme iron content was measured by the 2, 2′-bipyridyl method using $FeCl_3$ as a standard.[28]

Western Blot Analysis of Heme Oxygenase-1

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli[29] using 10%

polyacrylamide. Proteins separated by SDS-PAGE were blotted onto a nitrocellulose membrane. After incubation with anti-heme oxygenase-1 (HO-1) antibody (StressGen Biotechnologies Corp., Victoria, BC, Canada), the membrane was incubated with peroxidase-conjugated anti-rabbit IgG. HO-1 on the membrane was detected with a chemiluminescence kit (Amersham Life Science, Cleveland, OH, USA).

Lipid Peroxidation

The levels of lipid peroxidation (LPO) of liver subcellular fractions were measured using an LPO-586 Assay kit (Oxis International Inc., Portland, OR, USA). The assay principle is based on the reaction of a N-methyl-2-phenylindole with malonaldehyde (MDA) and 4-hydroxyalkenals (4-HNE).[30] The levels of LPO were expressed as MDA+4-HNE (nmol/mg protein).

Statistical Analysis

All data were expressed as mean ± S.E.M. Statistical analysis of data was performed using the two-tailed Student t-test after the F-test.

RESULTS

Changes in Food Intakes and Body Weights

Changes in food intake and body weights of the LA-treated LEC rats are shown Figure 1A and Figure 1B, respectively. LA at the 100 mg/kg dose had no effect on food intake and body weights gain in normal F344 rats (data not shown). There were no differences in the changes in food intake between any of the treatment groups until day 21. In the control group, a marked decrease in food intake due to the onset of acute hepatitis was seen from day 22. LA treatment at doses of 30 and 100 mg/kg suppressed this marked decrease in food intake. In comparison with the control group, the LA-treated groups administered doses of 100 and 30 mg/kg showed greater increases in body weight from days 8 and 17, respectively. As could be expected from the results of changes in food intake, a marked decrease in body weight was observed in the control group from day 22. LA treatment at doses of 30 mg/kg and 100 mg/kg suppressed the loss of body weight associated with the onset of hepatitis.

Serum Biochemical Parameters Reflecting Liver Injury and Hemolysis

To ascertain the effect of LA treatment on the liver injury in LEC rats, we measured serum AST and ALT activities, and T-Bil and TP concentrations (Table I). Hemolytic anemia is observed in LEC rats with hepatitis, just as it is in human patients with WD.[31] Free Cu ions released from the inflamed liver are thought to be involved in hemolysis. Therefore, we also evaluated the effect of LA treatment against the Cu-induced hemolysis (Table I). LA treatment at doses of 10 and 30 mg/kg slightly suppressed the increase in AST activity compared with the control group. Though the LA-treated group administered the 100 mg/kg dose showed a statistically significant decrease in AST activity, the activity was still markedly higher than that in Wistar rats. Although LA-treated group at the 10 mg/kg dose showed slight but significant increase in ALT activity, this increase was not observed in groups administered the higher doses of LA. Interestingly, LA treatment exerted a marked suppressive effect on increases in T-Bil concentrations in a dose-dependent manner. Decreases in TP concentrations were suppressed by LA in a dose-dependent manner. LA treatment suppressed hemolytic index values in a dose-dependent manner, although effects of LA were not significant.

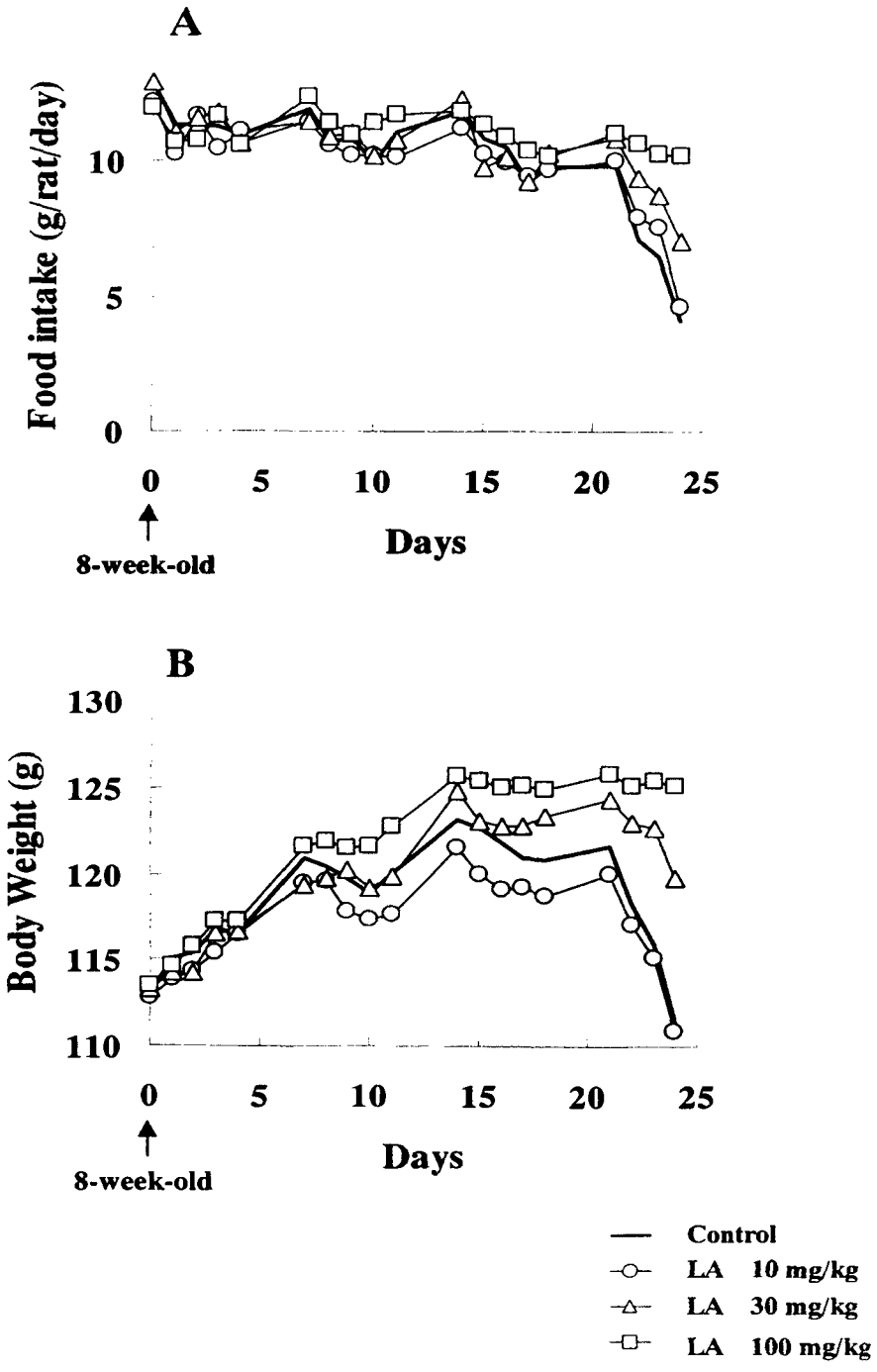

FIGURE 1 Changes in food intake and body weight in LA-treated LEC rats. Food intake (A), and body weight (B). Data indicates mean of seven rats for each treatment group. LA was administered orally five times per week in doses of 10, 30, and 100 mg/kg. Control rats were given vehicle alone. This was started with when the rats were 8 weeks old and continued until they were 12 weeks old (for 4 weeks), when jaundice was observed in the control group

TABLE I Serum biochemical analysis and hemolytic index in LA-treated LEC rats

Treatment	*AST (IU/L)*	*ALT (IU/L)*	*T-Bil (mg/dL)*	*TP (g/dL)*	*Hemolytic index OD (576–650)nm*
Wistar	111.5 ± 10.1	35.8 ± 1.5	0.04 ± 0.01	6.0 ± 0.1	
Control	1034.1 ± 81.1	829.3 ± 35.0	27.6 ± 3.6	4.0 ± 0.1	0.0208 ± 0.0106
LA 10 mg/kg	894.3 ± 61.0	1101.1 ± 56.1[a]	18.5 ± 5.2	4.3 ± 0.2	0.0107 ± 0.0052
LA 30 mg/kg	825.3 ± 98.4	973.0 ± 90.7	11.6 ± 4.3[a]	4.5 ± 0.2[a]	0.0067 ± 0.0015
LA 100 mg/kg	732.3 ± 101.0[a]	916.7 ± 58.1	1.4 ± 0.5[a]	4.9 ± 0.1[a]	0.0074 ± 0.0007

Values are indicated as mean ± S.E.M. for seven (LEC) or five (Wistar) rats.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin; TP, total protein.

a. significantly different from control group (*P<0.05, P<0.01)

TABLE II Antioxidant enzyme activities in liver cytosolic (CYT), crude mitochondrial (CMT) and 700g supernatant fractions from LA-treated LEC rats

Treatment	*Wistar*	*Control*	*LA*		
			10 mg/kg	*30 mg/kg*	*100 mg/kg*
CYT					
SOD	20.2 ± 1.0	15.9 ± 0.8[a]	16.8 ± 0.4	16.3 ± 0.9	14.9 ± 0.6
GPX	1.634 ± 0.050	0.653 ± 0.108[a]	0.824 ± 0.067	0.900 ± 0.072	1.110 ± 0.070[b]
GSSG-R	37.8 ± 3.6	97.9 ± 8.9[a]	141.3 ± 8.0[b]	123.8 ± 9.9	117.6 ± 7.3
CMT					
SOD	5.2 ± 0.3	5.2 ± 0.4	6.0 ± 0.3	6.2 ± 0.5	6.1 ± 0.7
GPX	0.353 ± 0.011	0.204 ± 0.020[a]	0.278 ± 0.016[b]	0.285 ± 0.016[b]	0.364 ± 0.033[b]
GSSG-R	11.9 ± 1.0	32.5 ± 2.3[a]	41.3 ± 1.6[b]	39.4 ± 3.2	42.4 ± 3.3[b]
700g supernatant					
CAT	544.1 ± 24.1	241.7 ± 23.1[a]	337.7 ± 38.1	390.5 ± 28.1[b]	526.5 ± 16.5[b]

Values are indicated as mean ± S.E.M. for seven (LEC) or five (Wistar) rats.
SOD, superoxide dismutase; GPX, glutathione peroxidase; GSSG-R, glutathione reductase; CAT, catalase. The units of SOD, GPX, GSSG-R and CAT activities are all expressed as units/mg protein.

a. significantly different from Wistar rats (##P<0.01)
b. significantly different from control group (*P<0.05, **P<0.01)

TABLE III Copper and nonheme iron contents in liver cytosolic (CYT) and crude mitochondrial (CMT) fractions from LA-treated LEC rats

Treatment	*Copper (nmol/mg protein)*		*Nonheme iron (nmol/mg protein)*	
	CYT	*CMT*	*CYT*	*CMT*
Wistar	6.6 ± 1.6	4.3 ± 0.8	8.4 ± 3.8	13.5 ± 5.1
Control	49.1 ± 8.0[a]	37.6 ± 8.8[a]	17.0 ± 8.2[a]	45.8 ± 7.1[a]
LA 10 mg/kg	51.7 ± 6.4	30.6 ± 4.9	12.2 ± 8.5	35.7 ± 12.3
LA 30 mg/kg	55.4 ± 4.9	30.1 ± 6.4	10.5 ± 5.3	32.1 ± 13.6[b]
LA 100 mg/kg	52.7 ± 3.7	25.5 ± 5.5[b]	8.4 ± 3.8[b]	22.9 ± 8.3[b]

Values are indicated as mean ± S.E.M. for seven (LEC) or five (Wistar) rats.

a. significantly different from Wistar rats (#P<0.05, ##P<0.01)
b. significantly different from control group (*P<0.05, **P<0.01)

TABLE IV Malonaldehyde plus 4-hydroxyalkenal contents in liver microsomal (MS) and crude mitochondrial (CMT) fractions from LA-treated LEC rats

Treatment	*MS (nmol/mg protein)*	*CMT (nmol/mg protein)*
Wistar	1.977 ± 0.116	0.290 ± 0.016
Control	3.545 ± 0.348[a]	1.224 ± 0.097[a]
LA 10 mg/kg	3.116 ± 0.251	1.250 ± 0.114
LA 30 mg/kg	2.478 ± 0.116[b]	1.098 ± 0.087
LA 100 mg/kg	2.344 ± 0.197[b]	0.895 ± 0.081[b]

Values are indicated as mean ± S.E.M. for seven (LEC) or five (Wistar) rats.

a. significantly different from Wistar rats (##P<0.01)
b. significantly different from control group (*P<0.05, **P<0.01)

Antioxidant Enzyme Activities in the Liver Cytosol (CYT), Crude Mitochondria (CMT) Fractions, and 700g supernatant fractions

Tables II show the SOD, GPX, and GSSG-R activities in the liver CYT and CMT fractions and the CAT activities in the liver 700g supernatant fraction from LA-treated LEC rats and Wistar rats. In CYT fraction, SOD activities in the control group were slightly but significantly lower than those in Wistar rats. We could not see any effect of LA treatment on the SOD activity. GPX activities in the control group were significantly lower than those in the Wistar rats. LA treatment suppressed the decrease in GPX activities in a dose-dependent manner. The GPX activities in the group received 100 mg/kg of LA were significantly higher than those in the control group. In contrast with the results of the previous two enzymes, GSSG-R activities in the control group were about threefold higher than those in Wistar rats. LA treatment at the 10 mg/kg dose significantly increased the GSSS-R activity, but this increase was not dose dependent.

In CMT fraction, SOD activities in the control group were almost similar to those in Wistar rats, while LA treatment had no effect on these activities. AS with the results in CYT fraction, LA treatment suppressed the decrease in the GPX activities in a dose-dependent manner. The GPX activities in the group received 100 mg/kg of LA were almost the same levels as those observed in Wistar rats. The effects of LA treatments on GSSG-R activities in CMT fractions were similar to the results observed in CYT fraction.

CAT activities in the control group were markedly lower than those in Wistar rats. LA treatment suppressed the decrease in the CAT activities in a dose-dependent manner. The CAT activities in LA 100 mg/kg-treated group were almost the same as those in Wistar rats.

Cu and Nonheme Iron Contents

It has been reported that LA and/or DHLA chelate transition metals such as Cu[21] and iron.[20] Therefore, we measured Cu and nonheme iron contents in liver CYT and CMT fractions (Table 3). As reported previously,[3,4] Cu contents in the control group were markedly higher than those in Wistar rats in both fractions. While LA had no effect on the Cu content in the CYT fraction, LA treatment at the 100 mg/kg dose significantly reduced the Cu content in the CMT fraction, though not nearly to a level low enough to approach that in Wistar rats.

Nonheme iron contents in both fractions were also higher in the control group than those in Wistar rats. In contrast with the results on Cu contents, LA treatment reduced nonheme iron

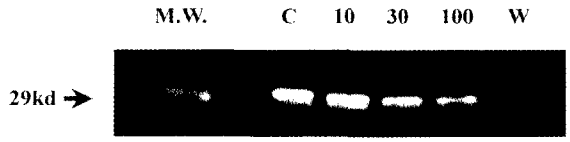

FIGURE 2 Western blot analysis of heme oxygenase-1 (HO-1) in liver microsomes from LA-treated LEC rats. The pooled (n=7 for LEC rats, n=5 for Wistar rats) microsomal proteins (25 μg/lane) were electrophoretically separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Blots were reacted with anti-rat HO-1 antibody and then visualized by the ECL. We confirmed the reproducibility of this result through repeated experiments. M. W., molecular weight markers; C, control LEC rats; 10, LEC rats treated with LA at a dose of 10 mg/kg; 30, LEC rats treated with LA at a dose of 30 mg/kg; 100, LEC rats treated with LA at a dose of 100 mg/kg; W, Wistar rats

contents in a dose-dependent manner in both fractions. In CYT fraction, LA treatment at the 100 mg/kg dose reduced the nonheme iron contents to almost the same level as those in Wistar rats.

Heme Oxygenase-1 (HO-1) Expression

HO-1 has been identified as the major 32-kd heat shock protein hsp32.[32] Like other stress proteins, HO-1 can be highly induced by a variety of seemingly disparate stimuli, most of which are linked by their ability to provoke oxidative stress.[33] We measured HO-1 expression to assess the extent of oxidative stress in the liver after LA treatment (Figure 2). In the livers of Wistar rats, we could not detect HO-1 expression. As expected, HO-1 was highly induced in the control group while its expression was suppressed in the LA-treated groups at the higher dose levels.

Lipid Peroxidation (LPO) in Microsomal (MS) and CMT Fractions

We measured the LPO as malonaldehyde (MDA) plus 4-hydroxyalkenals (4-HNE) in MS and CMT fractions to ascertain the effect of LA treatment against peroxidative cell damage (Table 4). LPO levels in both fractions were significantly higher in the control group than in Wistar rats. The degree of difference in LPO levels between the control group and Wistar rats was much greater in the CMT fraction. In the MS fraction, LA treatment at the doses of 30 and 100 mg/kg significantly reduced LPO levels, and the level in the LA-treated group administered the 100 mg/kg was almost the same as that in Wistar rats. In the CMT fraction, LA treatment at the 100 mg/kg dose significantly reduced the LPO level, but the level was still about threefold higher than that in Wistar rats.

DISCUSSION

LA has recently gained considerable attention as an antioxidant.[14,22] It has been shown that LA and/or its reduced form DHLA exerts ROSs quenching,[15–19] metal chelating[20,21] and antioxidant regenerating activity.[34,35] Administration of LA has been shown to be beneficial in a number of oxidative stress models such as ischemia-reperfusion injury, diabetes, cataract formation, and radiation injury.[14] Although positive results of LA were observed in animal models of liver diseases, including alcohol-induced damage and metal and CCl_4 poisoning, studies in humans using low doses of LA often show discrepancies and contradictions.[36] Recent studies and clinical trials on diabetic neuropathy[37] have shown that beneficial antioxidant effects of LA can be attained at relatively high doses (e.g. 600 mg/day). Therefore, the LA at high doses is expected to be useful as a therapeutic agent for liver diseases involving oxidative stress. In this study we investigated protective and antioxidant effects of LA against acute hepatitis in LEC rats, an animal model of WD and Cu-induced oxidative hepatic injury.

LA treatment at dose levels of 30 mg/kg and higher suppressed the loss of body weight and the decrease in food intake due to the onset of

hepatitis (Figure 1). While LA treatment at the 100 mg/kg dose suppressed the decrease in serum TP contents, it failed to suppress the increases in serum AST and ALT activities, two parameters that reflect hepatic cell destruction (Table I). It was reported that (R)-lipoic acid treatment to aging rats improved indices of metabolic activity as well as lowered oxidative stress in the liver.[38]This report, together with our results suggest that LA treatment ameliorated hepatic function while exerting no apparent improvement in the values of serum AST and ALT. However, LA exerted a suppressive effect on the increase in serum T-Bil concentration in a dose dependent manner. Hemolytic anemia is observed in LEC rats with hepatitis, just as it is in human WD patients.[31] It was reported that the free Cu concentration in the serum increased in LEC rats with hepatitis, together with signs of hepatic damage.[39] These free Cu ions in the circulation induce hemolytic anemia, possibly by changing erythrocyte membrane permeability or causing osmotic fragility.[40] In our study, LA treatment decreased serum hemolytic index values in a dose-dependent manner (Table I). LA was found to form a stable complex with Cu^{2+} and to decrease Cu^{2+}-induced ascorbate oxidation and LPO.[21] LA-induced prevention of Cu-catalyzed erythrocyte membrane degradation and subsequent hemolytic anemia may be one possible reason for the dose-dependent reduction in T-Bil concentration. Moreover, LA treatment suppressed the hepatic induction of HO-1 (Figure 2), a rate-limiting enzyme of bilirubin biosynthesis, which is known as a typical protein induced by oxidative stress.[33] This suppressed expression of HO-1 by LA treatment might also be responsible for the dose-dependent reduction in T-Bil concentration.

Sokol *et al.*[41] indicated that oxidative damage to mitochondria may be involved in hepatic Cu toxicity of patients with WD. The previous electron microscopy study also showed that certain mitochondrial changes in LEC rats resembled those encountered in the hepatocytes of patients with WD.[42] Therefore, we evaluated the antioxidant effects of LA treatment on mitochondria as well as cytosolic and microsomal fractions. LA treatment protected decreases in GPX activities in both CYT and CMT fractions, total CAT activity in a dose-dependent manner. It was reported that administration of Cu ions to rats produced oxidative stress and caused depletion of CAT and GPX in the liver.[43] Therefore, it is considered that LA or DHLA exerted an antioxidant effect on inactivation of CAT and GPX, two enzymes which are important for scavenging hydrogen peroxide.

LA could not reduce the Cu content in the CYT fraction, but at a dose of 100 mg/kg it slightly but significantly suppressed the accumulation of Cu. On the other hand, LA treatment reduced nonheme iron content in a dose-dependent manner in both CYT and CMT fractions. LA has been shown to form stable complex with Cu^{2+}[44] and, DHLA has also been shown to chelate Cu^{2+} *in vitro*.[45] It was also shown that LA treatment did not increase the biliary excretion of Cu.[46] Before the onset of hepatitis, Cu in the liver of LEC rats was mostly bound to metallothionein (MT) as a Cu^{+}-MT form.[47] Apparently, LA and DHLA do not have the capacity to remove Cu from MT in CYT fraction and promote the excretion of free Cu from the hepatocyte. Although 140-kDa form of Wilson's disease protein (ATP7B) was found to be localized to mitochondria,[48] the mechanisms of Cu transport from cytoplasma to mitochondria remains unclear. Cu^{+}-GSH was found to be a potential physiological Cu^{+} carrier for MT.[49] Mitochondrial GSH pool is derived from the activity of a mitochondrial transporter that translocates GSH from cytosol into the mitochondrial matrix.[50] Assuming that Cu is transported from cytoplasma into mitochondria in a form of Cu^{+}-GSH through a mitochondrial GSH transporter, competition between GSH and LA (and/or DHLA) for chelating Cu may be involved in the reduction of Cu content in CMT fraction seen after LA treatment at the dose of

100 mg/kg. In nonheme iron content, it has been shown *in vitro* that DHLA can remove iron stored inside ferritin by complexing it in the ferric form.[20] Thus, removal of ferritin-bound iron by DHLA might cause reduction of nonheme iron content in both CYT and CMT fractions.

In LEC rats, enhanced LPO is thought to be a more likely cause for the toxic action of copper.[51,52] In consistent with previous results,[51,52]LPO levels were significantly higher in both MS and CMT fractions from control LEC rats compared with those from Wistar rats. The increase in the LPO level in the CMT fraction was more marked than that in the MS fraction. Although our CMT fractions contain lysosomes and peroxisomes in addition to mitochondria, our results are similar to the findings in the patients with WD.[41] LA treatment almost completely suppressed the increase in the LPO level in the MS fraction at the dose of 100 mg/kg. While LA treatment at the same dose showed a slight suppressive effect on the increase in the LPO level in the CMT fraction, the level was still about three times higher than that in Wistar rats. Our previous report[52] suggests that hydroxyl radicals generated by Fenton-type reaction between hydrogen peroxide and free Cu play a crucial role for increase in LPO in post-mitochondrial supernatant fraction of livers from hepatitic LEC rats. As mentioned above, LA can form a stable complex with Cu^{2+} and decrease Cu^{2+}-induced LPO.[21] LA and DHLA also can scavenge hydroxyl radicals[16] and peroxyl radicals.[18] Added to these, our present study showed that LA treatment prevented the inactivation of CAT and GPX, two enzymes which are important for scavenging hydrogen peroxide. Therefore, it is considered that these antioxidant properties of LA and/or DHLA might contribute to the dose-dependent suppression of the increase in LPO in MS fraction. Although the mechanism of enhanced LPO in hepatic mitochondria have not been elucidated in the patients with WD and LEC rats, it was reported that mitochondrial Cu concentrations correlated strongly with the severity of mitochondrial LPO in the liver of patients with WD.[41] In our study, LA treatment at doses of 10 and 30 mg/kg had no effects on LPO and Cu content in CMT fraction. However, a slight but significant reduction of LPO in CMT fraction seen after LA treatment at the dose of 100 mg/kg was accompanied by the decrease in Cu content. Thus, reduction of Cu content by LA treatment was thought to be involved in the decrease in LPO in CMT fraction.

As mentioned above, enhanced LPO in mitochondria due to the Cu accumulation might play a crucial role for the development of liver damage in the patients with WD. Therefore, our results on LPO and Cu content in CMT fraction suggest that almost no effect of LA against the increases in serum AST and ALT activities might be linked to its inability, even at the highest dose, to reduce LPO and Cu content in the CMT fraction to the level found in the Wistar rat. However, a significant reduction of LPO and Cu contents in CMT fraction observed at the highest dose of LA supports an idea that LA has a potential to protect against the development of Cu-induced liver damage in LEC rats. In this study, we used the racemic form of lipoic acid. It was reported that (R)-form of lipoic acid more effectively chelated Cu and prevented Cu-induced LPO than (S)-form of lipoic acid.[21] Therefore, we expect that if we had used the (R)-lipoic acid treatment in this study, it would have reduced the mitochondrial copper more efficiently and exert stronger antioxidant effects against Cu-induced oxidative hepatic injury in LEC rats than the racemic form used

In summary, although LA treatment had little effect in reducing serum AST and ALT activities, LA treatment improved the clinical conditions caused by acute hepatitis. Furthermore, LA treatment prevented the development of liver damage at the molecular level, as evidenced by suppressive effects against inactivation of CAT and GPX and increases in LPO, Cu and nonheme iron content, and HO-1 expression. Our present findings using LEC rats, an animal model of

WD, suggest that treatment with a Cu-chelating agent supplemented with (R)-lipoic acid would be a more effective treatment strategy for WD patients than Cu chelating agent alone.

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