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Impaired Hepatic Copper Homeostasis in Long-Evans Cinnamon Rats: Reduced Biliary Excretion of Copper

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ABSTRACT. To explain the pathogenesis of excessive copper accumulation in Long-Evans Cinnamon (LEC) rats, regarded as one of the animal models for hepatic-type Wilson's disease, we measured copper contents in liver tissue and bile, serum total copper concentration, and ceruloplamin oxidase activity in LEC rats before and after the onset of spontaneous hepatitis. The copper contents in liver tissue of both 11-wk-old and 18-mo-old LEC rats were about 60 times the amounts in age-matched Wistar and Long-Evans Agouti rats. The biliary copper excretion in 11-wk-old LEC rats was significantly lower than that of the Long-Evans Agouti and Wistar rats that were the same age (27.9 and 41.4%, respectively). In 18-mo-old LEC rats, biliary copper excretion was lower than that in the Long-Evans Agouti rats that were the same age, but the finding was statistically not significant. Serum copper and ceruloplasmin levels were markedly reduced in LEC rats of both ages. These findings suggest that LEC rats have similar defects of biliary copper excretion as observed in patients with Wilson's disease. (*Pediatr Res* 35: 598–601, 1994)

Abbreviations

LEC, Long-Evans Cinnamon
LEA, Long-Evans Agouti
AST, aspartate aminotransferase
ALT, alanine aminotransferase
LDH, lactate dehydrogenase

Wilson's disease, a rare inherited disorder that results in copper toxicity, is autosomal and recessive and is characterized by high copper content in liver tissue, markedly reduced ceruloplasmin and low copper level in serum, and increased urinary copper excretion (1, 2). Although the precise mechanism is not clear, the basic defects of copper metabolism in Wilson's disease are a failure of biliary excretion of copper (3–5) and a reduced incorporation rate of copper into apoceruloplasmin (6). A recent report using computer simulation of copper metabolism in human beings also confirms that the primary defect in Wilson's disease is the sequestering of unavailable copper in the liver (7). A variety of experimental animals with autosomal and recessive inherited disorders, such as copper-loading Bedlington terriers (8), toxic milk mice (9), wild mute swan (10) and toads (*Bufo marinus* L) (11), have been extensively studied as a model of Wilson's disease because of their excessive copper accumulation in the liver. However, significant pathologic, histochemical, chemical, and clinical differences exist between each of these animals and the patients with Wilson's disease. The LEC rat is an inbred strain of mutant rats that were originally isolated in 1985 from a closed colony of Long-Evans rats (12). This strain, associated with spontaneous hepatitis (12, 13), exhibits excessive hepatic copper accumulation and lower copper level and ceruloplasmin oxidase activity in serum (14–19). Because of the similarities of its clinical and biochemical characteristics to the hepatic-type Wilson's disease, the LEC rat is regarded as one of potential animal models of Wilson's disease (18). However, data are limited regarding biliary copper excretion in LEC rats (16, 17). To further explain the pathogenesis of excessive copper accumulation in the liver of LEC rats, we determined the changes in biliary copper excretion as well as serum and hepatic copper contents in LEC rats before and after the onset of spontaneous hepatitis.

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MATERIALS AND METHODS

Animals. Male LEC and LEA rats at the 11th wk and 18th mo of age were maintained under the following conditions: lights on from 0700 h to 1900 h, temperature 23.0 ± 1.0°C, humidity 55 ± 5%. Water and food (CE-2, Clea, Tokyo, Japan) were given *ad libitum*. The number of animals and their body weights are shown in Table 1. LEC and LEA rats, two inbred strains selected for their coat color, were kindly provided by Dr. Noritoshi Takeichi, Hokkaido University, Sapporo, Japan, and bred in the Department of Pharmacology, Toho University School of Medicine, Tokyo, Japan. Wistar rats at the age of 10 wk were obtained from a commercial breeder (Sankyo Labo Service, Co., Tokyo, Japan) and served as control animals together with LEA rats. This study was performed in accordance with the guideline for animal experiments of the Toho University School of Medicine.

Methods of collecting bile and blood samples. All animals were anesthetized with 50 mg/kg body wt of sodium pentobarbital intraperitoneally and were fixed supinely on an operating board. A small, 2-cm flank incision was made at the skin area under the xiphoide process, and the common bile duct was exposed and then cannulated with polyethylene catheters (PE10, Nippon Becton Dickinson Co. Ltd., Tokyo, Japan). Bile was collected by draining spontaneously for 1 hr. After the bile was collected, blood was drawn from the carotid artery and immediately centrifuged (3000 rpm, 10 min, 4°C), and the sera were separated. The amount of serum and bile samples used for this study was 0.1 mL. The rat was killed by exsanguination via the carotid artery. After bile and blood were collected, the whole liver was removed, blotted, and weighed. Approximately 0.1–0.2 g of the liver tissue was used for measurement of copper content.

Copper content in serum, bile, and liver tissue and total biliary acid concentration in bile. Serum, bile, and liver tissue were wet ashed with nitric acid and exposed to a series of dilutions with distilled water. Copper content was analyzed using an inductively coupled plasma–mass spectrometer method (ELAN 5000, Perkin

Table 1. *Serum copper, ceruloplasmin, and liver-derived enzymes in Wistar, LEA, and LEC rats**

	11 wk old			18 mo old	
	Wistar	LEA	LEC	LEA	LEC
n	5	5	5	3	4
Body weight (g)	335 ± 3†	316 ± 9†	184 ± 9	507 ± 31‡	346 ± 27
Copper concentration (mg/L)	0.78 ± 0.02†	1.25 ± 0.11†	0.10 ± 0.03	1.47 ± 0.18§	0.56 ± 0.06†
Ceruloplasmin oxidase activity	0.16 ± 0.002‖	0.29 ± 0.04†	0.01 ± 0.003	0.24 ± 0.03§	0.05 ± 0.02‖
AST (IU/L)	135 ± 6	242 ± 43	469 ± 123	319 ± 122	443 ± 171
ALT (IU/L)	61 ± 6	93 ± 18	396 ± 147	214 ± 149	358 ± 175
LDH (IU/L)	1841 ± 267	2936 ± 301	2011 ± 397	5375 ± 869	4513 ± 1192

* Values are expressed as mean ± SEM. Ceruloplasmin oxidase activity is expressed as Δ OD/40 min.
† $p < 0.01$ *vs* 11-wk-old LEC.
‡ $p < 0.05$ *vs* 18-mo-old LEC.
§ $p < 0.01$ *vs* 18-mo-old LEC.
‖ $p < 0.05$ *vs* 11-wk-old LEC.

Elmer, Norwalk, CT). Total biliary acid in bile was measured by the enzymatic method (20). Biliary copper excretion was expressed as copper content per liter of bile (mg/L) and also as a ratio of copper content and also as a ratio of biliary copper content to total biliary acid in bile (mg/mmol).

Serum ceruloplasmin concentration. Serum ceruloplasmin concentration was measured as serum oxidase activity with a modified method of colorimetric enzymatic assay originally described by Ravin (21). Sodium acetate buffer (0.1 M, pH 4.0) and sodium azide in distilled water (0.3%) were prepared and stored at 4°C. Paraphenylendiamine sodium (0.5%) was prepared just before use. Serum sample (20 μL) was added to 100 μL of 0.5% paraphenylendiamine sodium in 0.1 M sodium acetate buffer (100 μL) in duplicate. To inhibit serum enzyme activity, we added 100 μL of 0.3% sodium azide to one of two tubes; it was then mixed and used as a control. Another sample was immediately incubated for 40 min at 37°C, and then 0.3% sodium azide (100 μL) was added and diluted with 500 μL of distilled water. The oxidase activity was measured with a spectrophotometer (A560, Hitachi Co., Tokyo, Japan). Paraphenylendiamine and sodium azide were purchased from Iwai Kagaku Co. (Tokyo, Japan).

Activities of serum AST, ALT, and LDH. Serum concentration of AST, ALT, and LDH were determined by the UV method with a Hitachi autoanalyzer (736-60E, Hitachi Co).

Statistical analysis. All values were expressed as mean ± SEM. Differences between means for all data were evaluated with the nonpaired *t* test. A *p* value of 0.05 or less was considered statistically significant.

RESULTS

There were no clinical symptoms of acute hepatitis, including jaundice, anemia, oliguria, and s.c. bleeding, in 11-wk-old LEC rats. However, the mean body weight was significantly lower than that of LEA and Wistar rats (Table 1). The LEC rats at 18 mo of age were still smaller than the LEA rats that were the same age and manifested no neurologic symptoms. Macroscopic findings of the liver of 18-mo-old LEC rats were compatible with those of hepatocellular carcinoma, whereas no macroscopic changes were observed in the liver of 11-wk-old rats. Serum AST and ALT levels of the LEC rats were higher than those of LEA rats at either 11 wk or 18 mo of age. No significant difference in LDH level were observed between each group of 11 wk-old rats, whereas the mean LDH levels in both LEA and LEC rats at 18 mo were elevated and higher than those of 11 wk-old rats (Table 1).

Serum total copper concentration and ceruloplasmin oxidase activity. Serum total copper concentration in the LEC rats at 11 wk of age was significantly lower than that in the LEA and Wistar rats (8 and 12.8%, respectively). The mean level increased at 18 mo of age but was still lower (38.1%) than that of the LEA rats that were the same age (Table 1). Serum ceruloplasmin oxidase activity of 11-wk-old LEC rats was markedly lower than that of LEA and Wistar rats (3.4 and 6.2%, respectively). Although the activity increased at 18 mo, the mean value remained significantly lower than that of LEA rats (20.8%). The LEA rats showed neither significant change in serum copper concentration nor ceruloplasmin oxidase activity (Table 1).

Copper content in liver tissue. Copper content in the liver of 11-wk-old LEA rats was similar to that of Wistar rats. However, the tissue content in LEC rats was approximately 60 times that in LEA rats at both 11 wk and 18 mo of age. No significant difference in copper content was observed between 11-wk-old and 18-mo-old LEC rats (Table 2).

Biliary copper excretion. Biliary copper concentration was significantly lower in 11-wk-old LEC rats when compared with those of LEA and Wistar rats that were the same age 27.9 and 41.4%, respectively). At 18 mo the copper concentration in LEC rats were still lower than that in the LEA rats that were the same age (47.5%), although the mean value became higher than that in 11-wk-old LEC rats (Fig. 1). Biliary copper excretion expressed by the ratio of biliary copper concentration to total biliary acid was lower in 11-wk-old LEC rats than that in LEA rats that were the same age. However, at 18 mo of age, the difference in biliary copper excretion was not significant between LEC and LEA rats (Table 2).

DISCUSSION

Acute hepatitis occurs in 90% of LEC rats between 16–23 wk of age. The serum AST level exceeds 1200 IU, the ALT level reaches approximately 500 IU, and about 30% of LEC rats die of acute hepatic failure (13). Chronic hepatitis or hepatocellular carcinoma may develop in rats between 12–15 mo of age that have survived (22), as seen in the 18-mo-old rats in the present

Table 2. *Copper levels in liver and bile**

	n	Copper content in liver (μg/g wet tissue)	Biliary copper excretion†
11 wk old			
Wistar	5	3.1 ± 0.2‡	1.93 ± 0.58
LEA	5	3.3 ± 0.8‡	1.64 ± 0.27§
LEC	5	186.7 ± 21.27	0.68 ± 0.13
18 mo old			
LEA	3	4.4 ± 0.1‖	0.10 ± 0.03
LEC	4	234.0 ± 42.6	0.15 ± 0.03

* Values are expressed as mean ± SEM.
† Ratio of biliary copper concentration to total biliary acid in bile ($\times 10^{-2}$ mg/mmol).
‡ $p < 0.01$ *vs* 11-wk-old LEC.
§ $p < 0.05$ *vs* 11-wk-old LEC.
‖ $p < 0.01$ *vs* 18-mo-old LEC.

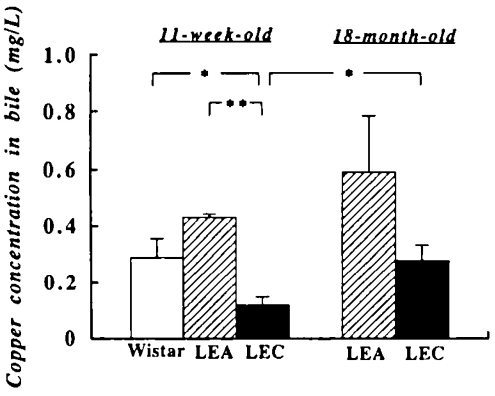

Fig. 1. Biliary copper concentration in LEC, LEA, and Wistar rats at 11 wk and 18 mo of age. *, $p < 0.05$; **, $p < 0.01$.

study. The 11-wk-old LEC rats we used were clinically regarded as being at the stage shortly before the onset of acute hepatitis, although serum liver-derived enzyme activities were slightly elevated and we did not examine the liver histologically. Marked copper accumulation in the liver and low serum copper and ceruloplasmin concentrations were recognized in these 11-wk-old LEC rats. These findings are consistent with previous reports (14–19). According to the ontogenic studies on copper metabolism in LEC rats (14, 18), hepatic copper content in LEC rats is already three times that in LEA rats at the 2nd day of age and increases from the 4th postnatal wk, reaches the highest level (about 90 times) at the 16th week, and then decreases and remains at the 40 times higher level until the 8th mo, which is the oldest age studied (18). Although conflicting data exist regarding serum copper and ceruloplasmin levels in 2-d-old LEC rats (14, 18), serum copper and ceruloplasmin concentrations after the neonatal period are reported to be 10–38% and 0.6–19% of those in age-matched LEA rats, respectively, until 8 mo of age (14, 18). When fulminant hepatitis occurs, the serum copper level exceeds that of LEA rats and decreases if the rat survives (18). We observed the same characteristics of copper metabolism in 11-wk-old LEC rats that we did in 18-mo-old LEC rats that had survived acute hepatitis and thereafter developed chronic hepatitis or hepatocellular carcinoma. The absolute values of serum copper and ceruloplasmin levels increased in these rats compared with 11-wk-old rats. Although the exact reason is obscure, a similar change is reported in the LEC rats between 3 and 8 mo of age (14).

In addition, we confirmed that biliary copper excretion was markedly reduced in both 11-wk-old and 18-mo-old rats, providing further support regarding their similarities to the hepatic type of Wilson's disease. The ratio of biliary copper content to total biliary acid was lower in the presymptomatic young patients with Wilson's disease (average age, 8 y) studied in our laboratory. We observed this ratio was markedly lower in 11-wk-old LEC rats than that in LEA and Wistar rats. However, the difference became statistically not significant at 18 mo, although the value was still lower than that in younger rats. The reason for this finding is not clear and needs further investigation.

Ceruloplasmin is a single-chain polypeptide synthesized in the membrane-bound polyribosome (23), and it incorporates copper within the lysosomes of the hepatic cells. In plasma, more than 90% of the copper is bound to ceruloplasmin, which exists as a holoprotein bound to six or seven copper atoms per molecule. Although the ceruloplasmin gene and Wilson's disease gene are located on different chromosomes (24, 25), serum ceruloplasmin levels in about 95% of patients with Wilson's disease are diminished or absent because of decreased ceruloplasmin gene transcription (26), subsequently causing a reduced incorporation rate of copper into apoceruloplasmin. With MAb against ceruloplasmin, we detected the inactive ceruloplasmin that was lacking oxidase activity and binding activity to copper in serum from the patients of Wilson's disease even when their serum ceruloplasmin levels were markedly low (27). This finding suggests the possibility of impaired conversion to active ceruloplasmin in patients with Wilson's disease. We and others have also confirmed the presence of apoceruloplasmin in serum of LEC and LEA rats by Western blotting (19, 28).

The precise mechanism of copper homeostasis in the liver is not fully understood (6). Most of copper actively absorbed from the intestine is loosely attached to albumin in blood, transported to the liver, and taken up into the hepatic cell by not well-defined mechanisms. Amino acid or albumin, forming copper-binding complex, is the candidate for this transport across the plasma membrane. Copper is then transferred to cellular particles for the generation of copper proteins, such as ceruloplasmin, cytochrome oxidase, superoxide dismutase, and tyrosinase. Copper is distributed throughout the subcellular fraction of the hepatocyte. Within the lysosome, copper is prepared for biliary excretion. When hepatic copper overload progresses, as in Wilson's disease, the excess copper is stored in an insoluble form in the lysosome. Recently, much attention has focused on defective mechanisms of copper transport within the hepatocytes in pathogenetic factors of Wilson's disease. For example, impaired copper transport across lysosomal or canalicular membranes and enhanced copper retention by a lysosomal protein are speculated (2).

Several hypotheses have been proposed to explain the defective biliary copper excretion in Wilson's disease. They include *1*) synthesis of abnormal protein with high copper binding activity (29), *2*) persistent fetal mode of copper metabolism (30) on the basis of the concept of a lysosomal defect in hepatocytes, *3*) absence or defect of the putative carrier proteins in the hepatocyte required for incorporating copper into ceruloplasmin or excreting copper by way of the bile (6, 31), and *4*) deficiency of copper-binding protein in bile (32–34), probably the protein identical to serum ceruloplasmin (34). It is also reported that normal human bile contains two molecular forms of ceruloplasmin. One is identical to the serum ceruloplasmin, and the other is similar to the ceruloplasmin without oxidase activity detected in patients with Wilson's disease (35).

In conclusion, our results provide additional evidence that a close similarity in clinical and biochemical findings exists between LEC rats and patients with Wilson's disease, suggesting that LEC rats can be an animal model that may serve to explain the mechanisms of Wilson's disease.

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