Biliary excretion of copper in LEC rat after introduction of copper transporting P-type ATPase, ATP7B

Kunihiko Terada[a], Namiko Aiba[a], Xiao-Li Yang[a], Masatake Iida[a], Michio Nakai[b], Naoyuki Miura[a], Toshihiro Sugiyama[a,*]

[a] *Department of Biochemistry, Akita University School of Medicine, Hondo 1-1-1, Akita 010-8543, Japan*
[b] *Discovery Research Laboratories III, Sumitomo Pharmaceuticals Research Center, 3-1-98 Kasugadenaka, Konohanaku, Osaka 554-0022, Japan*

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Abstract Wilson's disease, an autosomal recessive disorder, is characterized by the excessive accumulation of hepatic copper that results from reduced biliary copper excretion and disturbed incorporation of copper into ceruloplasmin. The *ATP7B* gene, responsible for the disease, encodes a copper transporting P-type ATPase. We previously demonstrated the involvement of ATP7B in hepatic copper secretion into plasma after the introduction of *ATP7B* into the Long-Evans Cinnamon (LEC) rat, a rodent model of Wilson's disease. In this study we found the increased copper contents of the hepatic lysosomal fractions and bile in the LEC rats after *ATP7B* introduction, indicating the participation of ATP7B in the biliary excretory pathway for copper.

Key words: Copper transport; ATP7B; Bile; Wilson's disease; Long Evans Cinnamon rat

1. Introduction

Although copper is an essential trace metal, copper in excess of cellular needs is toxic [1]. Patients with Wilson's disease and its rodent model, the Long-Evans Cinnamon (LEC) rat, are characterized by the excessive accumulation of hepatic copper and manifest similar pathologic features including the reduced biliary excretion of copper, reduced copper in plasma and a remarkable decrease of serum ceruloplasmin (CPN) oxidase activity [2–4]. The genes responsible for Wilson's disease and the LEC rat have been designated *ATP7B* (previously designated *WND*) and *atp7b*, respectively (for review see [5]). The abnormal copper metabolism in the affected liver is believed to be due to mutations of these genes. Indeed, numerous mutations including point mutations, deletions, frame-shifts and splice errors have been reported in the patients with Wilson's disease [6]. In the LEC rat, *atp7b* is known to be deleted at its 3′ end [7] and its expression is absent [8].

ATP7B or Atp7b is predicted to be a copper transporting P-type ATPase from the deduced amino acid sequence. P-type ATPase is phosphorylated at their aspartic acid residue in the DKTGT/S motif and translocates a variety of cations across membranes [9]. Among P-type ATPases, ATP7B or Atp7b is further classified as a heavy metal transporting P-type ATPase that pumps copper or cadmium [10,11]. The characteristic features of ATP7B are six metal binding motifs, GMTC*XX*C, at the N-terminus of the molecule (five motifs in Atp7b), the CPC motif in the sixth transmembrane region, the SEHPL motif and eight transmembrane segments. The metal binding motifs are specific for copper and 1 mol of the N-terminus containing six binding motifs of the protein binds to 6 mol of copper [12]. The CPC motif and the SEHPL motif, as well as the metal binding region, in ATP7B are critical for copper transport by this protein [13–15].

Major export pathways for copper from hepatocytes include its secretion into plasma following its incorporation into CPN and its excretion into bile. ATP7B is thought to play an important role in these processes. The importance of ATP7B in the intracellular transport of copper is supported by recent studies using a yeast strain lacking *CCC2*, the yeast homologue of *ATP7B* [13,14]. These studies have revealed that ATP7B is able to complement the function of CCC2. An in vitro study using a murine fibroblast cell line carrying defective Atp7a, another mammalian copper transporting P-type ATPase, has shown that ATP7B substitutes Atp7a function in copper export [15]. We recently demonstrated the restored secretion of holoCPN, oxidase active and copper bound form, following the introduction of *ATP7B* into the LEC rat by recombinant adenovirus mediated gene delivery [16]. This indicates that ATP7B functions in copper secretion into plasma, however, the role of ATP7B in the biliary excretion of copper has not been directly demonstrated.

In this study, we introduced *ATP7B* into the LEC rat by recombinant adenovirus mediated gene delivery and examined ATP7B function with respect to the biliary excretory pathway for copper.

2. Materials and methods

2.1. Animal studies

Inbred LEC rats were bred under specific pathogen-free conditions in the Animal Facilities for Experimental Medicine, Akita University School of Medicine. Animals had free access to water and standard rat chow. All experiments were performed in accordance with the animal guidelines of Akita University School of Medicine. Recombinant adenoviruses bearing full-length *ATP7B* cDNA, designated AxCAWD, were prepared as described previously [16]. 1×10^{10} plaque forming unit (pfu) of AxCAWD, or control virus AxCAwt, were administered to six or 11 week old LEC rats by tail vein injection. Blood was collected from the carotid vein before and 3 days after viral infusion. Plasma was stored at −80°C until use. Bile was collected 3 days after infusion in the following manner. Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight) and the bile duct was cannulated with polyethylene tube (PE-10, Igarashi, Tokyo). Liver was removed 3 days after infusion and weighed prior to homogenization.

*Corresponding author. Fax: (81) (18) 884 6443.
E-mail: sugiyama@med.akita-u.ac.jp

Abbreviations: LEC, Long-Evans Cinnamon; CPN, ceruloplasmin; pfu, plaque forming unit; PAGE, polyacrylamide gel electrophoresis

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Table 1
Copper distribution in the subcellular fractions of livers from the LEC rats after infusion of the recombinant adenovirus

	Cu (μg/g wet liver)			
	PNS	Microsome	Lysosome	Cystosol
+wt-1	114.1	2.2	0.14	75.5
+wt-2	101.3	1.8	0.18	70.9
(Mean)	(107.7)	(2.2)	(0.16)	(73.2)
+ATB7B-1	61.4	0.34	1.25	27.4
+ATB7B-2	61.6	0.58	2.10	18.9
(Mean)	(61.5)	(0.46)	(1.68)	(23.4)

Copper distribution in the subcellular fractions of livers from the LEC rats after infusion of the recombinant adenovirus. Four 6-week-old LEC rats were infused with 1×10^{10} pfu of either AxCAWD (+ATP7B-1, +ATP7B-2) or AxCAwt (+wt-1, +wt-2), the control virus. The subcellular fractions were prepared freshly from the rat livers 3 days after infusion as described in Section 2. The copper contents (μg/g wet liver) in the subcellular fractions were determined by atomic absorption spectrophotometry. PNS, postnuclear supernatants. The mean value of each fraction from two rats is also shown.

2.2. Preparation of subcellular fractions

The subcellular fractions of liver were obtained from the LEC rat infused with recombinant adenoviruses. The procedures described below were performed at 4°C. The liver was minced and homogenized in 3 volumes (w/v) of SHE buffer (0.25 M sucrose/10 mM HEPES-NaOH, pH 7.2/1 mM EDTA) using a Potter-Elvehjem homogenizer with a Teflon pestle at 1000 rpm for five strokes. The homogenate was centrifuged for 10 min at $600\times g$, and the resulting supernatant was saved as postnuclear supernatant. The postnuclear supernatant was centrifuged for 10 min at $5000\times g$, and the supernatant was recentrifuged for 10 min at $25\,000\times g$. The lysosomal fraction was prepared from the $25\,000\times g$ pellet by Percoll gradient centrifugation as described previously [16]. The $25\,000\times g$ supernatant was centrifuged for 1 h at $140\,000\times g$ and the resulting supernatant and pellet were saved as cytosol and microsomal fraction, respectively. The pellet containing the microsomal fraction was suspended in SHE buffer and homogenized with a Teflon pestle by hand. Enrichment of the lysosomal fraction was confirmed by immunodetection using antibodies against human cathepsin D (Upstate Biotechnology, Lake Placid, NY).

2.3. Western blot analysis

The protein samples were resolved by polyacrylamide gel electrophoresis (PAGE) under denaturing or non-denaturing conditions, and immunodetection was performed by Western blot analysis as described [17]. Antibodies against ATP7B and CPN were prepared as described previously [16].

2.4. Measurement of copper content

The copper contents in the subcellular fractions and bile were measured directly by atomic absorption spectroscopy using a polarized Zeeman atomic absorption spectrophotometer (Model 180-80, Hitachi, Japan) equipped with graphite furnace.

3. Results

3.1. Copper accumulation in lysosomal fractions of LEC rat livers after adenoviral infusion

To determine the intracellular distribution of copper after the introduction of *ATP7B*, the subcellular fractions of liver were prepared from the LEC rats 3 days after infusion of AxCAWD or control virus. As shown in Table 1, the mean value of lysosomal copper contents obtained from the two rats with introduced *ATP7B* was 10.5-fold higher than that of two controls (1.68 μg/g wet liver vs. 0.16 μg/g wet liver), whereas the mean value of cytosolic copper contents in these rats was reduced to 32% of the controls (23.4 μg/g wet liver vs. 73.2 μg/g wet liver). The enrichment of the lysosomal fraction was confirmed by the immunoblotting using the antibody against cathepsin D known as a lysosomal marker (Fig. 1, lower panel). In the rats in which *ATP7B* was introduced, the accumulated copper in the lysosomal fraction seems not to be sequestered in association with this protein in this organelle, since ATP7B is not detected in this fraction, but in the microsomal fraction which contains Golgi apparatus (Fig. 1, upper panel), the site where ATP7B resides [13,16,18].

3.2. Increased excretion of copper into bile after adenoviral infusion

To investigate ATP7B function with respect to the functional biliary excretory pathway for copper, copper contents in bile collected from the LEC rats 3 days after viral infusion were determined. Since the bile duct in the LEC rat less than 200 g of body weight is thin compared to the normal rat, 11-week-old LEC rats (250 g of body weight) were used. As shown in Fig. 2, the two LEC rats with introduced *ATP7B* showed 2.4-fold increase of biliary copper content relative to the two controls (the mean values are 2.63 ng/min vs. 1.10 ng/min). The holoCPN secretion into plasma in these two rats with introduced ATP7B was confirmed by the immunodetection (Fig. 3, upper panel), is consistent with our recent study [16]. No holoCPN was secreted in the two control rats (Fig. 3,

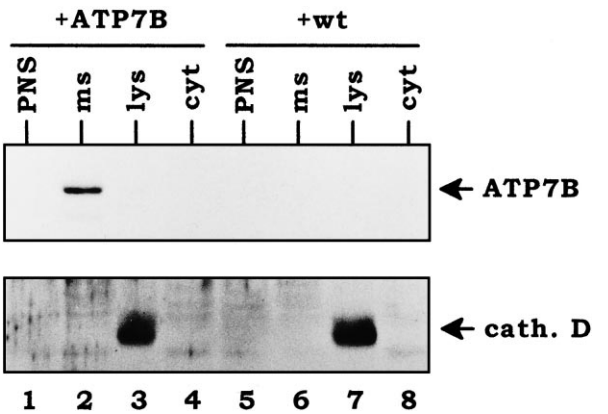

Fig. 1. Expression of ATP7B in the subcellular fractions of livers from the LEC rats after infusion of the recombinant adenovirus. Western blot analysis of equivalent amounts of protein samples (5 μg) obtained from the subcellular fractions of the LEC rat livers after infusion with either AxCAWD (+ATP7B, lanes 1–4) or AxCAwt (+wt, lanes 5–8). The subcellular fractions were identical to those used in Table 1. The protein samples were subjected to 7% SDS-PAGE and transferred to a PVDF membrane. The blot was probed with anti-ATP7B monoclonal antibody (1:200, upper panel), and anti-human cathepsin D polyclonal antibody (1:200, lower panel). Bound antibodies were detected by chemiluminescence. Lanes 1 and 5, post nuclear supernatants (PNS); lanes 2 and 6, microsomal fractions (ms). Lanes 3 and 7, lysosomal fractions (lys). Lanes 4 and 8, cytosol (cyt). A representative experiment is shown.

lower panel). These results indicate that the biliary excretion of copper and the secretion of copper were restored in the LEC rats after the introduction of *ATP7B*.

4. Discussion

In the present study we demonstrated the restoration of biliary copper excretion in the LEC rats after the introduction of human *ATP7B* cDNA using the recombinant adenovirus.

The major export pathways for hepatic copper consist of the secretion into plasma following its incorporation into CPN and the excretion into bile. Since ATP7B is predicted to be a copper transporting P-type ATPase from its amino acid sequence, ATP7B is suggested to play an important role in these processes. Indeed, our recent study has revealed that ATP7B is involved in the secretion of copper coupled with CPN synthesis [16], although the association of ATP7B with the biliary export pathway for copper was only indirectly demonstrated [19]. To resolve this problem, we examined the intracellular distribution of copper and the recovery of biliary excretion of copper in the LEC rats after the introduction of *ATP7B*. Consequently, we found the decrease of cytosolic copper, the elevated levels of copper content in the lysosomal fractions and the increased biliary excretion of copper in the LEC rats infused with AxCAWD, not with control viruses. The cytosolic copper was probably reduced by its discharge into bile through the lysosomal fractions. These indicate that ATP7B functions in the excretory pathway for copper, as well as in the secretory pathway. These data are consistent with prior studies on experimental copper overload states in rat liver [20] and studies on Wilson's disease patients [21] suggesting that lysosomes were the sites for storage of copper and the main source of biliary copper. Additionally, evidence that biliary excretion of copper was inhibited by a microtubule inhibitor, colchicine, indicated the involvement of vesicular transport in the biliary copper export [22]. Based on these data and observations that ATP7B localizes to the Golgi apparatus in hepatocytes [13,16,18], we hypothesize that the excretory route for copper from hepatocytes is as follows: cytosolic copper taken up into the Golgi apparatus by ATP7B is transported to lysosomes by vesicular transport. Subsequently, copper in lysosomes is released into bile by exocytosis. Since the LEC rat exhibits the spontaneous accumulation of copper in the liver, resembling experimental copper overload states, a considerable amount of copper seems to be transported through the lysosomal-biliary pathway after the induction of ATP7B.

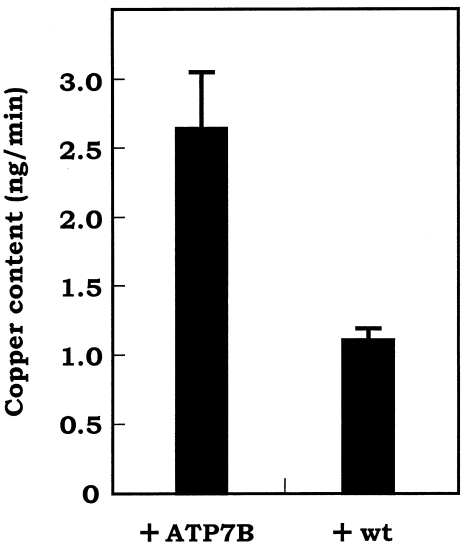

Fig. 2. Copper contents in bile obtained from the LEC rats after infusion of the recombinant adenovirus. Bile samples were obtained from four 11-week-old LEC rats infused with 1×10^{10} pfu of recombinant viruses. The two LEC rats were infused with AxCAWD (+ATP7B), and the other two with AxCAwt (+wt), the control virus. The samples were collected 3 days after viral infusion. The copper contents were determined by atomic absorption spectrophotometry. The mean value of each group of rats is shown.

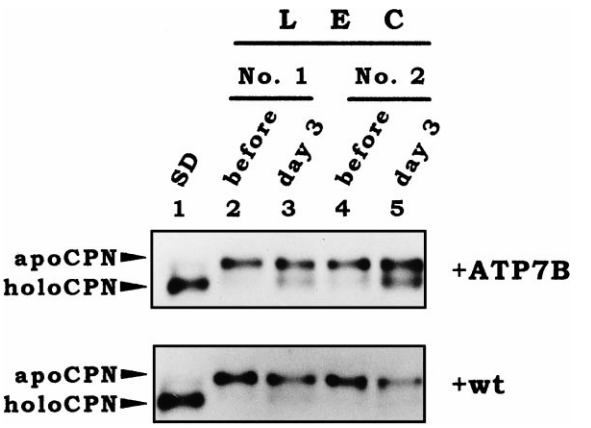

Fig. 3. Detection of holoceruloplasmin in plasma of the LEC rats after infusion of the recombinant adenoviruses. Western blot showing the chemiluminescent detection of ceruloplasmin in plasma of the LEC rats. Plasma samples were obtained from four 11-week-old LEC rats infused with 1×10^{10} pfu of either AxCAWD (upper panel, +ATP7B (1 and 2) or AxCAwt, the control virus (lower panel, +wt (1 and 2). The samples were subjected to 7% PAGE under non-denaturing conditions and transferred to PVDF membranes. The blots were probed with anti-rat ceruloplasmin antibodies (1:1000). Lane 1, plasma from Sprague-Dawley (SD) rat. Lanes 2 and 4, plasma from LEC rats before viral infusion. Lanes 3 and 5, plasma from LEC rats 3 days after viral infusion. Plasma taken from the SD rat and the LEC rats were diluted to 1:500 and 1:100, respectively, and 10 μl of each was applied. Plasma from the SD rat was applied to show the mobility of holoceruloplasmin on PAGE under non-denaturing conditions.

The requirement of ATP7B in hepatic copper transport explains the pathogenesis of Wilson's disease. Patients with the disease have a defect in *ATP7B*, leading to a disturbance of intracellular copper transport. Among numerous mutations of ATP7B reported in the patients, mutations of H1069Q or N1270S were shown to lose their copper transport function in vitro studies [13–15]. In addition, mutations introduced into the critical sites in ATP7B, such as the DKTGT/S motif and the CPC motif, were also found to alter the copper transport function of ATP7B [13,14]. These observations imply that intact ATP7B is necessary to regulate copper homeostasis in the liver. In the intracellular trafficking of copper, ATP7B may act as a gate which controls the copper flow through the secretory and excretory pathways in liver.

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