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Abnormal Accumulation of Porphyrin Derivatives in the Kidneys of Long-Evans Cinnamon Rats, as Evidenced by Microspectrophotometry

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In the study described here we have revealed an abnormal accumulation of porphyrin derivatives in the kidneys of Long-Evans Cinnamon (LEC) rats, an animal model for human Wilson's disease. In addition, we have confirmed that the derivatives emitted red-orange light in renal sections under UV excitation. This renal red-orange emission has previously been identified as luminescence from cuprous metallothioneins [Cu(I)-MTs], which also accumulate in both the kidneys and liver of LEC rats. In this study, we measured the emission spectra of the luminescence in the kidneys using microspectrophotometry. The spectra of the renal red-orange emission resembled those of porphyrin derivatives rather than those of Cu(I)-MTs. We then extracted these derivatives from the kidneys. An abundance of porphyrin derivatives was established. A significant increase in the levels of the derivatives in the liver and urine of the LEC rats was also confirmed. These results provide evidence of a heme-metabolism abnormality in LEC rats.

Key Words: **microspectrophotometry; luminescence; porphyrin derivatives; metallothioneins; copper; LEC rat; Wilson's disease.**

Wilson's disease (hepatolenticular degeneration) is an autosomal recessive disorder of copper (Cu) transport that is characterized by failure to incorporate Cu into ceruloplasmin in the liver, and failure to excrete Cu from the liver into bile. This results in the toxic accumulation of Cu in the liver and kidneys (see reviews 1-4). The Long-Evans Cinnamon (LEC) rat is an animal model for Wilson's disease (5-9). An abnormal accumulation of Cu in the liver and low concentrations of both ceruloplasmin and Cu in the serum have been demonstrated to occur in these rats. This accumulation of Cu is explained by the defective expression of the Cu-transporting P-type ATPase gene, which is homologous to the gene for Wilson's disease (*ATP7B*) (10-12).

The age-dependent accumulation of Cu in the LEC rat has been reported to occur not only in the liver but also in the kidneys, resulting in damage to these organs (5, 6, 8, 13). Most of the Cu that accumulates in these organs was discovered to be captured by metallothioneins (MTs) (14) as cuprous MTs [Cu(I)-MTs] (15, 16). MTs are low-molecular-weight proteins that bind unusually high amounts of heavy metal ions such as Cu, Zn, Cd and Hg (17, 18). It is well known that when illuminated with UV light at room temperature Cu(I)-MTs emit visible yellow-orange light at around 600 nm (19-23). Recently, using this unique property of Cu(I)-MTs, the localization of these proteins was visualized in the liver (24) and kidneys (25) of LEC rats. The hepatic yellow-orange luminescence that occurs in 15-week-old LEC rats has been identified as an emission from Cu(I)-MTs using three indispensable histochemical criteria based on the coordination chemical characteristics of MTs, as follows: (i) extinguishment due to oxidation of Cu(I) ions to cupric [Cu(II)] ions, (ii) quenching by protonation to dissociate Cu(I) ions bound to MTs, and (iii) vanishment due to the displacement of Cu(I) ions bound to MTs by Hg ions. The renal red-orange and yellow-orange emissions that occur in 30-week-old LEC rats were also verified as luminescence from Cu(I)-MTs with quenching tests using a Cu(I) ion chelating reagent and Hg ions, and with evidence of the colocalization of cysteine residues or immunocytochemistry for MTs.

In this study, we tried to characterize these emissions in renal and hepatic sections of 30-week-old LEC rats by using microspectrophotometry. The spectral properties of the renal red-orange emissions resembled

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those of porphyrin derivatives rather than those of Cu(I)-MTs, although the renal and hepatic yellow-orange emissions were completely explained as specific luminescence from Cu(I)-MTs. We then extracted the derivatives from the kidneys of the LEC rats. An abundance of porphyrin derivatives was established. Therefore, we conclude that the renal red-orange emission indicates an abnormal accumulation of porphyrin derivatives, and not of Cu(I)-MTs.

MATERIALS AND METHODS

Chemicals. Tris hydroxymethyl aminomethane (Tris) and Entellan neu were obtained from Sigma Chemical Co. (St. Louis, MO) and Merck (Darmstadt, Germany), respectively. Coproporphyrin, uroporphyrin and protoporphyrin fluorescence standards were purchased from Porphyrin Products (Logan, UT).

Animals. Thirty-week-old male LEC rats were used in this study. Age-matched Long-Evans Agouti (LEA) male rats were utilized as controls. Animals from the domestic line of Hokkaido University and from Charles River Japan (Tokyo, Japan) were used for this study. They were kept on a laboratory diet and tap water *ad libitum*, and were housed in a facility that was maintained at 22°C with a 12-h light-dark cycle. All procedures were carried out on laboratory rats according to the regulations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animals were perfused transcardially with a solution of 40 mM Tris-20 mM HCl containing 152 mM NaCl (500 ml/kg) after being administered an overdose of pentobarbital anesthesia (60 mg/kg, intraperitoneally injected). Their livers and kidneys were quickly removed, frozen with liquid nitrogen, and stored at −80°C until they were analyzed.

Microspectrophotometric analysis. The preparation of sections was performed as described in our previous report (24). We chose Entellan neu as a rapid embedding medium to avoid quenching the luminescence, since the medium possesses characteristics of low absorbance at the 300 nm region, faint emission at the visible region, high chemical stability under UV excitation, and because it enables the use of easily recyclable quartz cover slips and slides. Cu(I)-MTs for the emission spectrum *in vitro* were obtained from 30-week-old LEC rat livers and kidneys as described in our previous report (26), but with slight modifications. After gel filtration of the cytosol using a Superdex 75 column HR 10/30 (Pharmacia Biotech, Uppsala, Sweden), the fractions containing Cu(I)-MTs were pooled and then rechromatographed using the same column. The main single peak of absorbance and Cu concentration was collected and utilized in this study (data not shown). For the microspectrophotometric analysis of Cu(I)-MTs *in vitro,* Cu(I)-MTs were precipitated with cold acetone and embedded with Entellan neu.

A microscope photometer (MPM 800; Carl Zeiss, Oberkochem, Germany) was used for the measurements. It was equipped with a 100-W Hg-xenon high-pressure lamp. By selecting the appropriate filter units (an excitation filter of 300 ± 30 nm for spectrum scanning, or of 340 ± 30 nm for photographic recording, a 400-nm dichroic mirror and a 420-nm barrier filter), the specimens were viewed, and the emission spectra of the luminescence were recorded in the visible region from 500 to 700 nm at room temperature. All of the microspectrophotometric experiments were performed within 6 h of the embedding. All of the spectra shown in this study were corrected using the blank spectrum of the embedding medium.

Porphyrin analysis in tissues and urine. The porphyrin derivatives in the livers and kidneys were extracted as total porphyrins according to a modification of the method of Abbritti and De Matteis (27, 28). The frozen tissues were thawed and homogenized in 20 volumes of 1.0 M perchloric acid-methanol (1:1, v/v) at 20,000 rpm for 1 min on ice with a Polytron (PTA 10TS-TI; Kinematica AG, Luzern, Switzerland). The homogenate was centrifuged at 10,000 × g for 10 min at 4°C. The emission spectrum of the obtained supernatant between 500 and 700 nm under excitation at 405 nm was recorded as the extracted fraction including total porphyrins with the use of a fluorescence spectrophotometer (Model F-3000; Hitachi, Ltd. Tokyo, Japan).

The levels of porphyrin derivatives in the urine of the LEC or LEA rats were also measured as total porphyrins by using the method described above. The urine was collected into a foil-wrapped 50-ml polypropylene tube with a metabolic cage (Lab Products Inc., Maywood, NJ) for 24 h. The collected urine was centrifuged at 10,000 × g for 10 min at 4°C. Methanol (5.0 ml) and 10 M perchloric acid (0.50 ml) were added to the urinary supernatant (5.0 ml). After mixing well, the mixtures were re-centrifuged at 10,000 × g for 10 min at 4°C. The emission spectrum of the obtained supernatant between 500 and 700 nm under excitation at 405 nm was also recorded as the fractions including total porphyrins.

The spectral property of the obtained supernatant was compared with three standard porphyrins (i.e., coproporphyrin, uroporphyrin and protoporphyrin) dissolved in 1.0 M perchloric acid-methanol (1:1, v/v). The concentration of total porphyrins in the tissues and urine was then determined by the method of standard additions by adding appropriate amounts of the porphyrin that was identical with the spectral property of the obtained supernatant. The excitation wave-

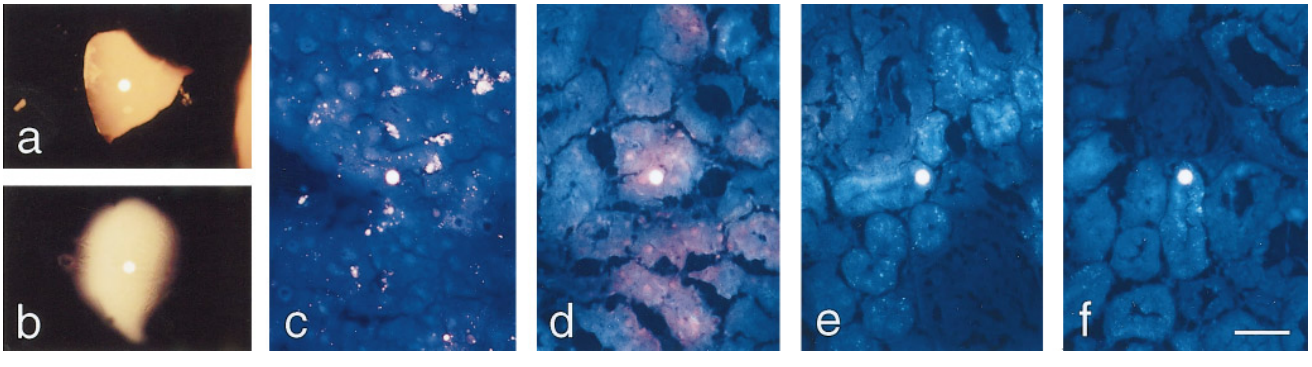

FIG. 1. Photomicrographs of luminescence from acetone-precipitated cuprous metallothioneins [Cu(I)-MTs] from a 30-week-old Long-Evans Cinnamon (LEC) rat liver (a) and kidney (b). Also shown are photomicrographs of yellow-orange emissions in a liver section (c), and red-orange light (d) and yellow-orange light (e) in a renal section from a 30-week-old LEC rat. Fig. 1f shows a photograph of the same region of an age-matched Long-Evans Agouti (LEA) rat kidney as shown in Fig. 1e. White spots in the photographs indicate measurement points (a diameter of 10 μm) for the emission spectrum analysis shown in Fig. 2a and b. Magnification, ×100. Bar = 50 μm.

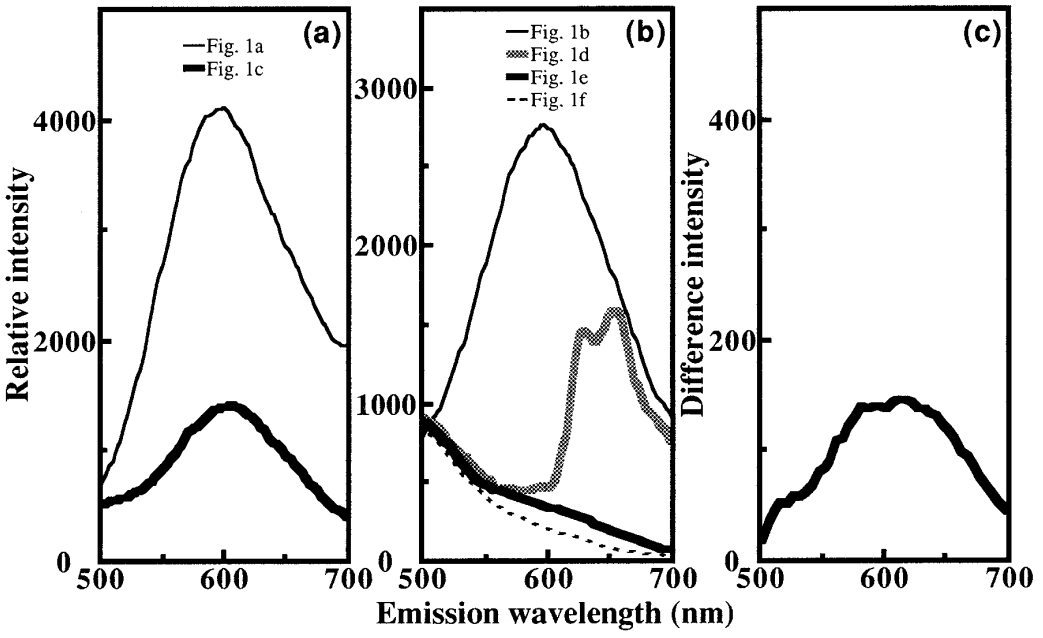

FIG. 2. (a) Emission spectra of the regions indicated by the white spots shown in Fig. 1a and c. (b) Emission spectra of the regions indicated by the spots shown in Fig. 1b, d, e and f. (c) Difference spectra between the yellow-orange light in the LEC rat kidney (Fig. 1e) and control emissions in the LEA rat kidney (Fig. 1f).

length was set at 405 nm and the emission was detected at 596 nm or 600 nm. The statistical significance of the difference between the results obtained for the LEC and LEA rat groups was evaluated by Student's t-test.

RESULTS

Microspectrophotometric analysis of sections. The yellow-orange luminescence from the Cu(I)-MTs obtained from the liver and kidney of a 30-week-old LEC rat is shown in Figs. 1a and b, respectively. The luminescence observations in the sections of the LEC rats are shown in Fig. 1c-e. In the livers of 30-week-old LEC rats, brilliant yellow-orange luminescence was also observed as described in our previous study (24). The emissions from the hepatic sections were observed in the cytoplasm and in some vacuolated nuclei of the hepatoparenchymal cells, and in the spherical granules (i.e., lysosome-like organelles) in both these and Kupffer cells. The most intense emissions were observed in the lysosome-like organelles. The luminescence satisfied completely the three indispensable histochemical criteria of Cu(I)-MTs (data not shown). In the kidneys of the LEC rats, strong ring-shaped red-orange emissions were observed predominantly in the outer stripe of the outer medulla. The emissions were found in the nuclei and cytoplasm of the proximal straight tubular cells of segment 3. Yellow-orange light was detected in lysosome-like organelles in the proximal convoluted tubule cells of segments 1 and 2, adjacent to the glomeruli in the cortex. These renal observations were exactly the same as those described in a previous report (25), and this luminescence also satisfied the quenching tests for Cu(I)-MTs (25).

The emission spectra of the emissions presented in Fig. 1 are shown in Figs. 2a and b. The emission maxima of both hepatic and renal Cu(I)-MTs *in vitro* were located at around 600 nm. These peaks were broad. The maximum of the yellow-orange luminescence in the liver sections was detected at around 600 nm, which was identical with that of Cu(I)-MTs *in vitro.* However, the red-orange emission in the renal sections consisted of two peaks at around 625 and 650 nm, respectively. The longest previously demonstrated emission maximum of Cu(I)-MTs was recorded at approximately 630 (29, 30) or 623 nm (31). No emission maximum of a wavelength longer than 630 nm has been reported. Moreover, the two renal peaks at around 625 and 650 nm were very sharp. Their spectral characteristics resembled those of porphyrin derivatives (32-39) rather than those of Cu(I)-MTs, both *in vitro* and in the liver. These results, therefore, indicate that the chemical species emitting red-orange light in the LEC rat kidneys were porphyrin derivatives, and not Cu(I)-MTs.

No distinct peaks of the yellow-orange emission in the proximal convoluted tubule cells of the renal cortex were detected. However, the emission intensity between 500 and 700 nm was stronger than that in the same areas in the renal sections of age-matched LEA male rats (a photograph of the measuring spot in the LEA rat kidney is shown in Fig. 1f). The difference

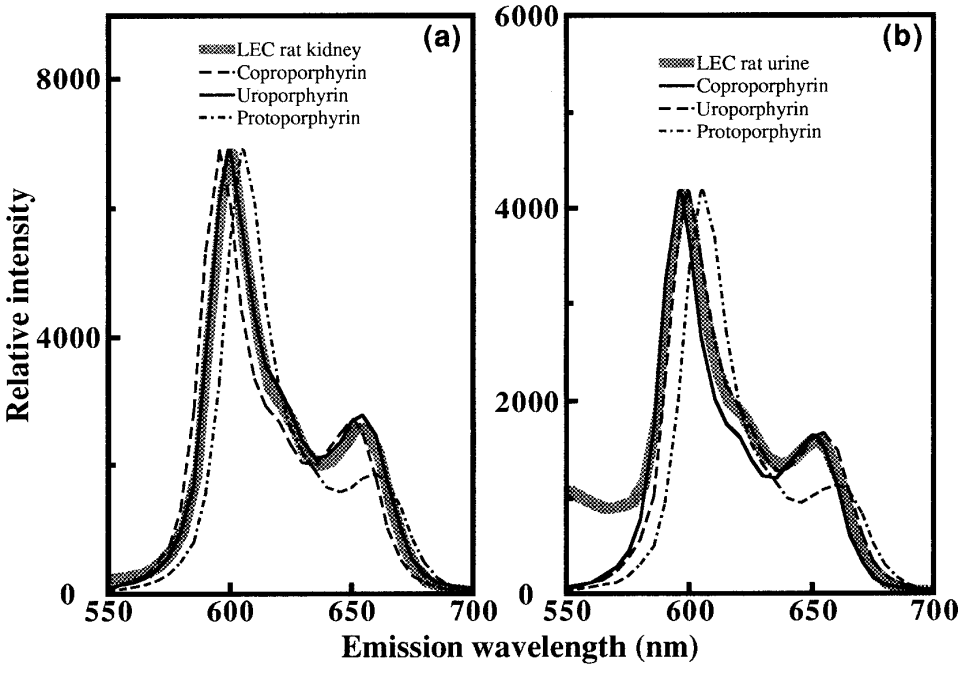

FIG. 3. (a) Comparison between the emission spectra of the total-porphyrin fraction extracted from a 30-week-old LEC rat kidney and of three standard porphyrins (i.e., coproporphyrin, uroporphyrin and protoporphyrin) under excitation at 405 nm. (b) Comparison between the emission spectra of the urinary extract of the LEC rat and of three standard porphyrins under excitation at 405 nm.

spectrum between the LEC and LEA rats was also analyzed (Fig. 2c). The spectrum clearly demonstrates the existence of a small emission maximum at around 600 nm from Cu(I)-MTs in the yellow-orange light.

Porphyrin analysis in tissues and urine. According to our microspectrophotometric data of the renal red-orange emission, we extracted porphyrin derivatives from the kidneys and livers. As in a previous report (40), the derivatives were detected as the emission between 580 nm and 700 nm under this condition. The characteristic of these emission peaks was nearly identical with that of uroporphyrin, and not coproporphyrin or protoporphyrin (Fig. 3a). We also determined the concentration of total porphyrins in the tissues using uroporphyrin fluorescence standard (Table I). A striking increase in the concentration of total porphyrins was revealed in the kidneys of the LEC rats. The concentration was about 19 times higher than that of age-matched LEA rat kidneys. Although the level of total porphyrins in the LEC livers was also elevated significantly, this hepatic concentration was about as low as that of the control rat kidneys. In addition, we determined the concentration of total porphyrins in the urine. Since the emission characteristic of the urinary extract was nearly identical with that of coproporphyrin, and not uroporphyrin or protoporphyrin (Fig. 3b), the concentration of total porphyrins in the urine was determined using coproporphyrin fluorescence standard. The concentration of total porphyrins in the urine of the LEC rats was also significantly higher than that of the control rat urine (Table I). These results, therefore, indicate an abnormal renal accumulation of porphyrin derivatives, mainly uroporphyrin, and a heme-metabolism abnormality in 30-week-old LEC rats.

DISCUSSION

We have established the existence of a heme-metabolism abnormality in 30-week-old LEC rats. In addition, we have confirmed that the red-orange emission that occurs in the LEC rat kidneys indicates an abnormal accumulation of porphyrin derivatives, and not of Cu(I)-MTs.

In a previous report (25), both the yellow-orange and

TABLE 1

Concentration of Total Porphyrins in the Kidney, Liver, and Urine of 30-Week-Old LEC and LEA Rats

Samples	LEC (n = 6)	LEA (n = 4)
Kidney (μg/g of wet tissue)	10.2 ± 5.47*	0.53 ± 0.02
Liver (μg/g of wet tissue)	0.64 ± 0.07**	0.23 ± 0.01
Urine (μg/day)	29.5 ± 3.29*	13.0 ± 0.70

Note. Each value represents a mean ± S.D. Significant differences from control values of LEA rats are indicated by *$p < 0.005$; **$p < 0.001$.

red-orange emissions observed in 30-week-old LEC rat kidneys were identified as specific luminescence from Cu(I)-MTs with the aid of several quenching tests, as well as biochemical, immunocytochemical and histochemical confirmation. In addition, the distribution of the red-orange emission was also in accord with the localization of MT mRNA. From those examinations, the renal red-orange emission of the LEC rat was believed to be that of abnormally accumulated Cu(I)-MTs. There is no doubt that abnormal accumulation of Cu(I)-MTs occurs in the LEC rat kidneys. However, our present results indicate that not only Cu(I)-MTs but also porphyrin derivatives are abnormally accumulated in the same region of the LEC rat kidneys, and that the porphyrin derivatives, not the Cu(I)-MTs, emitted red-orange light in the renal sections under UV excitation. This finding was revealed by our microspectrophotometric analysis of the emissions in the sections. Therefore, we emphasize the importance of examining the spectral properties of the emissions to avoid any serious mistake such as confusing porphyrin derivatives with Cu(I)-MTs.

The localization of the red-orange-emitting materials in the LEC rat kidneys was nearly identical to that previously demonstrated for accumulated porphyrins in renal porphyria (41-45). Although the mechanism of the accumulation of porphyrin derivatives in the LEC rat kidneys is unknown at present, we believe that there are three possible explanations. The first one is metal-induced porphyria (i.e., Cu-induced renal porphyria). This type of disorder is well-known to result from renal intoxication by lead, arsenic and inorganic or methyl Hg (41, 45-51). Acute intermittent porphyria as a result of Cu-poisoning has also been reported to occur (52). The second is a secondary phenomenon that occurs in the rats as a result of spontaneous hepatic injury. There are many reports on the interactions between hepatic injury and porphyria (44, 45, 53-56). It has been suggested that the mechanism of this type of disease depends upon faulty enzyme control in the hepatic biosynthesis of heme (55). The third possibility is that this accumulation of porphyrin derivatives is the result of a hereditary disease in the rat. We are currently investigating all of these possibilities.

The LEC rat is a promising animal model for Wilson's disease because these animals exhibit many clinical, biochemical and molecular-biological features that are similar to those which occur in the human disorder. In Wilson's disease, no case of renal porphyria has yet been demonstrated, although there has been one report on the disease complicating porphyria cutanea tarda (57). However, our results suggest that Wilson's disease might be accompanied by renal porphyria as a possible symptom in addition to the large accumulation of Cu, and that abnormal levels of porphyrin derivatives in the urine might make possible a new method of urinary diagnosis or screening for the disease. Therefore, we believe that the association of these two symptoms should be studied as a priority.

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