Copper metabolism in the kidney of rats administered copper and copper-metallothionein

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Kurasaki, Masaaki, Masashi Okabe, Shigeru Saito, and Mika Suzuki-Kurasaki. Copper metabolism in the kidney of rats administered copper and copper-metallothionein, Am. J. Physiol. 274 (Renal Physiol. 43): F783–F790, 1998.—To gain a greater understanding of the mechanism of Cu metabolism in kidneys of rats, using autofluorescence of Cu-metallothioneins (Cu-MTs) we revealed the behavior of Cu-MTs in the kidneys of rats administered Cu-MTs. Yellow and orange fluorescent signals of Cu-MTs were observed in the cortex. By microscopic studies, Cu-MTs were dominant in the proximal convolute tubular cells of the cortex, a high concentration of Cu-MT presented in the lysosome-like organs of the proximal convolute tubular adjacent to the glomeruli. During the time course after the injection, the orange signal in lysosome-like organs gradually converted to a yellow signal, indicating that the Cu-MT was involved in a degradation process in lysosomes by oxidation, and the MT mRNA increased in the cortex, although the immunoreactivity of MT was almost constant in the same region. These results suggested that Cu bound to the injected MT was released in lysosomes and became a new inducer of MT biosynthesis in the cortex. In conclusion, the biosynthesis and degradation of Cu-MT occur repeatedly in the proximal convolute tubular cells.

On the other hand, the signal was only detected in the cortex of kidney of macular mice (29). MT mRNA in both kidneys was also observed in the region where the fluorescent signals were detected, indicating that the MT having the fluorescence was biosynthesized in this region. The reasons for the difference in renal location between LEC rats and the macular mice are still unclear.

The different localizations of the Cu-MT revealed by the previous studies prompted us to investigate the renal Cu metabolism on the basis of Cu-MTs. MT is released from the liver into the bloodstream as a result of minor hepatic injuries (9) and is subsequently reabsorbed by the brush border of the proximal convoluted tubular (PCT) cells after the glomerular filtration in the kidneys (8). Although MT plays remarkable roles in the liver, the absorbed MT in the kidneys is toxic, due to free heavy metal ions that may be released from the MT by lysosomal degradation and/or by oxidation in the PCT system (7). On the other hand, Squibb et al. (27) suggested that MT in kidneys is not only taken up from blood but is also newly induced.

To gain insights into the mechanism of Cu metabolism in the tissue, we investigated the histochemical distribution of Cu-MTs in the kidney of rat administered with Cu-MT or Cu. The elucidation of its distribution in the kidney would greatly contribute to the understanding of the system for Cu transport, storage, and excretion and the mechanism of Cu and/or Cu-MT metabolism.

MATERIALS AND METHODS

Materials. Zeta probe membrane was obtained from Bio-Rad (Richmond, CA). Mouse anti-MT antibody (clone: E9, isotype: 1gG1k) was purchased from Zymed (San Francisco, CA). Colloidal-gold (10 nm)-conjugated streptavidin and species-specific biotinylated sheep anti-mouse IgG were obtained from Amersham International (Buckinghamshire, UK). We prepared Cu12-MTs from commercially available rabbit Cds2Zn2-MT (Sigma, St. Louis, MO) by the method described previously (16, 19). The digoxigenin DNA labeling and detection kit was from Boehringer Mannheim. Sephadex G-50 was obtained from Pharmacia Biotechnology (Upplands, Sweden). Other chemicals were reagent grade.

Animals. Twenty-one male Wistar rats (5 wk old, 120–140 g body wt) were bought from Japan SLC (Hamamatsu, Japan). The rats were fed ad libitum MF-4 standard pellets (Oriental Yeast) containing 1.5 mg of Cu/100 g and water and housed at a constant temperature (22 ± 2°C).

Cu or Cu-MT was administered to the rats intraperitoneally daily by injections of 1.5 mg of Cu/kg body wt as CuCl2 in 0.9% NaCl or 1.5 mg Cu-MT/rat (12 mg of Cu/kg body wt) in 10 mM Tris-5 mM HCl containing 100 mM NaCl. The total number of injections was three times per one rat. Each group
of three rats was received an overdose of pentobarbital anesthesia (60 mg/kg), 24, 48, and 120 h after the final injection, and each rat received transcardial perfusion with 40 mM Tris-20 mM HCl containing 152 mM NaCl (500 ml/kg). The kidneys were quickly removed and frozen with liquid nitrogen. All procedures were performed according to the regulations as defined by the NIH “Guide for the Care and Use of Laboratory Animals.”

Histochemical procedures. All procedures were carried out according to the methods of Okabe et al. (20) with some modifications. Zeta probe membranes were used to observe the overall kidney, and glass slides were used for the microscopic observations (section thickness: for membrane blotting, 20 µm; for glass slides, 5 µm). Membrane blotting was processed according to the method of Okabe et al. (21). Briefly, the obtained tissues were frozen and cut with a cryostat microtome. The sections were mounted on dry Zeta probe membranes and thawed at 25°C. The membranes were incubated on filter papers prewetted with a 40 mM Tris·HCl buffer, pH 8.0, for 10 min at 4°C. After the transfer, the tissues were removed by a high-pressure jet spray of the same buffer. Tissue-mounted glass slides were immersed in 70% ethanol. The fluorescent emissions of Cu-MT were photographed by using a Nikon FP-30 fluorescent microscope system with an U-MWU filter cube (DM-400 dichroic mirror; 385-nm excitation filter, 530-nm barrier filter) with a 100-W mercury lamp as an ultraviolet (UV) light source. The Cu-MT fluorescence has been proved to be essentially attributed to Cu-MT (17, 20). The fluorescence signals were observed in the cortex of the kidney at all time points after the injection. The order of the intensity of the fluorescent signals at each time point was estimated to be 24 h > 48 h > 120 h. The control rat did not exhibit fluorescent signals (data not shown) (20). The fluorescence has been proved to be essentially attributed to Cu-MT (17, 20).

The localization of immunoreactive MT (Fig. 3, A–D) showed a good analogy with those of autofluorescent emissions. The immunoreactivity of MT was also observed at all time points in the renal cortex of rats administered Cu-MT or Cu. We examined the histochemical localization of Cu-MT in the overall kidney of rats administered Cu-MT 24, 48, and 120 h after the final injection by autofluorescent observation of Cu(I)-thiolate clusters in Cu-MT (Fig. 2, A–C). The fluorescent signals were observed in the cortex of the kidney at all time points after the injection. The order of the intensity of the fluorescent signals at each time point was estimated to be 24 h > 48 h > 120 h. The control rat did not exhibit fluorescent signals (data not shown) (20). The fluorescence has been proved to be essentially attributed to Cu-MT (17, 20).

RESULTS

Cu and Zn contents of kidneys of rats administered Cu-MT or Cu. Cu contents in the kidney of rats administered Cu-MT dramatically increased 24 h after the final injection (Fig. 1A), and then the contents decreased 120 h after the injection. In contrast, Zn contents in the kidney were slightly higher than that of control rats and slightly increased during the time course (Fig. 1A).

Amounts of Cu and Zn contents in the kidneys of rats administered Cu also increased at all time points in comparison with those of control rat kidneys (Fig. 1B). However, Zn contents in the kidney slightly decreased during the time course (Fig. 1B).

Overall localization of Cu-MT in the kidney of rats administered Cu-MT or Cu. We examined the histochemical localization of Cu-MT in the overall kidney of rats administered Cu-MT 24, 48, and 120 h after the final injection by autofluorescent observation of Cu(I)-thiolate clusters in Cu-MT (Fig. 2, A–C). The fluorescent signals were observed in the cortex of the kidney at all time points after the injection. The order of the intensity of the fluorescent signals at each time point was estimated to be 24 h > 48 h > 120 h. The control rat did not exhibit fluorescent signals (data not shown) (20). The fluorescence has been proved to be essentially attributed to Cu-MT (17, 20).

The localization of immunoreactive MT (Fig. 3, A–D) showed a good analogy with those of autofluorescent emissions. The immunoreactivity of MT was also observed at all time points in the renal cortex of rats administered Cu-MT or Cu. We determined the genomic expression of MT in the kidney of rats administered Cu-MT or Cu. Briefly, 0.5 g kidney of rats administered with Cu-MT or Cu was homogenized (1:2, wt/vol) in 50 mM Tris·25 mM HCl and centrifuged at 9,000 g for 30 min. The supernatant was subsequently recentrifuged at 100,000 g for 1 h. The cytosols was applied to a column (1 × 40 cm) of Sephadex G-50, and the eluents were analyzed for metal concentrations and absorbance at 280 nm with a Hitachi frame atomic absorption spectrophotometer (model 180–80) and a Beckman spectrophotometer (model Du-65), respectively.

Determination of Cu and Zn contents in kidney of rat administered with Cu or Cu-MT. To determine Cu and Zn contents in kidneys of rat administered Cu and Cu-MT, 0.5 g of the tissue was digested with mixed acids (1 ml concentrated H2SO4, 5 ml concentrated HClO4, and 10 ml concentrated HNO3). The metals were analyzed with a Hitachi frame atomic absorption spectrophotometer (model 180–80).

Fig. 1. Cu (solid bars) and Zn (open bars) contents of kidneys of rat administered with Cu-metallothionein (Cu-MT) (A) and Cu (B); n = 3. Error lines are SD.
administered Cu-MT (Fig. 3, A–C). In the kidney of rats administered Cu, the fluorescent signal and immunoreactivity of MT were slightly observed in the cortex of the kidney 24 h after the injection (Figs. 2D and 3D) and 48 h and 120 h after the final injection (data not shown).

Genomic expression of MT. The expression of MT mRNA was investigated to examine whether the MT in the renal cortex was de novo biosynthesized during the time course after the administration of Cu-MT (Fig. 4, A–C). Although the expression of MT mRNA was hardly detected in the kidney 24 h after Cu-MT administration, it was observed in the cortex 48 and 120 h after the administration. The kidney of the control rat did not exhibit the expression of MT mRNA (data not shown). From these results, it was postulated that the Cu-MT transported into kidney by the bloodstream was degraded in the cortex, and the biosynthesis of MT occurred in this region by released Cu ions from Cu-MT administered.

Detailed localization of autofluorescent signals in the tissue. To determine the detailed histochemical localization of Cu-MT in the kidney, microscopic analyses under UV irradiation were carried out. The orange autofluorescent signals were observed exclusively in the PCT and near the glomeruli of the PCT segment S1 and S2 of the cortex (Fig. 5, A and B). In the whole tube, the fluorescence presented as a large cluster (Fig. 5A, arrows) and small particles (Fig. 5A, arrowheads). On the other hand, the fluorescence was scarcely observed in some tubes which were estimated as distal convoluted tubules (Fig. 5A, asterisks), glomerulus (Fig. 5B and 5C, white circle), and vascular endothelium (Fig. 5D, arrow). Yellow fluorescent signals appeared from 48 h after the injection of Cu-MT in the same region (Fig. 5C and 5D), indicating that Cu-MT was degraded in the organelles, such as the lysosomes. Yellow fluorescence increased in the regions 120 h after the Cu-MT administration; however, orange fluorescent signals were scarcely found in the region (Fig. 5, E and F). The fluorescent particles were found in the PCT (Fig. 5F, arrow) and aggregated in comparison with those 24 h and 48 h after the final injection (Fig. 5, E and F, arrowheads). Fluorescent emissions were not observed in the medulla and pelvis of the kidney (data not shown), even by microscopic observation. The kidney of rats administered with Cu hardly showed fluorescent signals in the tissue (Fig. 5G).

The yellow signals were concluded to be present in lysosomes because the yellow particle also exhibited acid phosphatase activity, which is a histochemical marker of lysosomes (data not shown) (20, 29). The granules of orange and yellow signals showed signs of Cu-MT, as both signals were abolished after the Hg(II) treatment (Fig. 5, H and I). It is well known that Hg-binding capacity of MTs is stronger than Cu-binding capacity of them and that Cu bound to MTs is displaced with Hg. Although in the cortex 24 h after the final injection, the histochemical aspects of the lysosome-like granules indicated the presence of Cu-MT, the expression of MT mRNA did not occur (Fig. 4A).

Purification of low-molecular-weight, Cu-binding protein from the kidney of rats administered Cu-MT. Figure 6, A to C, shows representative elution patterns of the Sephadex G-50 chromatography of the renal
cytosol from the rat at 24 h to 120 h after administration of Cu-MT. $K_d$ value (34) of the low-molecular-weight Cu peak in the chromatogram of a rat administered Cu-MT was in good agreement with that of the authentic Cd,Zn-MT (Fig. 6D). Thus the Cu-containing peak was considered to be Cu-binding MT. Zn content in the fraction of Cu-containing MT increased during the time course after the Cu-MT administration.

DISCUSSION

In this study, to gain the mechanism of the Cu metabolism in the kidney of rats, we revealed the
behavior and localization of Cu-MT in the kidney of rats administered with Cu-MT or Cu, using autofluorescence dependent on the Cu-S cluster in the Cu-MT. As the fluorescent spectra detected in vivo were reported to be similar to those observed from Cu-MT in vitro (28), the histochemistry of Cu-MT with use of autofluorescence signals in tissues has been studied in our laboratory (17, 20, 23, 29). Of course in animal tissues, several other biomolecules, e.g., vitamin A, lipofuscins, and porphyrins, emit autofluorescence under the UV excitation. We established the Cu-MT histochemistry using the protein-chemical aspects of MT, e.g., the existence of Cu(I)-thiolate clusters, immunoreactivity, and cysteine histochemistry as described in a previous report (20).

At first, we found that the Cu-MT injected in rats was transported to the cortex of kidneys (Fig. 3A) and emitted orange fluorescence (Fig. 2A). The substance having the yellow-orange signals was identified as Cu-MT, since the fluorescence in granules was abolished by treatment with Hg(II) (Fig. 5, H and I), as shown in previous reports (17, 20, 29).

From the result that MT mRNA was hardly detected in the cortex (Fig. 4A), it was thought that the emitted Cu-MT was not biosynthesized in the region. Yellow and orange fluorescent signals were observed in the lysosome-like organelles of PCT S1 and S2 adjacent to the glomeruli in the cortex (Fig. 5, A and B). The orange signal in lysosome-like organelles was gradually converted to a yellow signal within the duration of the time course (from 24 h to 120 h after the Cu-MT injection), indicating the Cu-MT was involved in the degradation process in lysosome-like organelles by oxidation (Fig. 5, A–F). The change in the fluorescence from orange to yellow was presumed to be the partial release of Cu ions and was suggestive of the oxidation of Cu-MT in the cortex, because the fluorescence of model complexes of Cu-MT changed from orange to yellow by decreasing the molar ratio of the Cu(I)thiolate group (I). We also demonstrated previously that the Cu-MT in the cortex is located in the lysosome-like organelles in LEC rats (20).

During the time course, although the fluorescent signal slightly decreased, the level of the MT mRNA
increased in the cortex (Fig. 4, A–C). The level of immunoreactivity against MT antibody was almost the same in the same region (Fig. 3, A–C). From these results, it was thought that Cu bound to the injected MT released in lysosome and became a new inducer of de novo biosynthesis of MT in the same region. The results of gel filtration (Fig. 6, A–C) also supported this consideration. Zn content in the MT fraction dramatically increased in the renal cytosol of rats administered Cu-MT 120 h after the injection in comparison with that of rats administered Cu-MT 24 and 48 h after the injection. Since it is well known that Cu binding ability to MT is stronger than Zn binding ability to the protein, the Cu bound to MT is hardly replaced with Zn in cells. The Cu,Zn-MT obtained from the renal cytosol 120 h after the administration was thought to be the de novo synthesized protein by Cu ions released from Cu-MT in lysosomes. As Cu-MT diffuses in the cytoplasm and/or the Cu-MT also bound Zn, the emission intensity in the cytoplasm could be relatively sparse and/or weak.

Microscopic studies of the detailed localization of Cu-MT showed that Cu-MT was dominant in the PCT cells of the cortex in rat (Fig. 5, A–F). These results correspond well with the immunohistochemistry of the protein in the kidneys of Cu-loaded rodents (10, 26, 33). Our results that Cu-MT administered into rats readily passes through the glomerulus are coincided with the reports described by many researchers (7–9, 27). Following glomerular filtration, the protein bound to the brush border of PCT cells and was taken up into these cells. Radioisotope-labeled cysteine residues of MT are also primarily confined to these cells (7). We postulate that the protein, which was transported from other organs, located in the cortex in normal rats, since MT mRNA was scarcely observed in this region 24 h after the administration of Cu-MT (Fig. 4A).

It has been reported that after chronic Cu administration, Cu-MT accumulates mainly in the PCT cells of the kidney (10, 26, 33). However, in this study, the fluorescence and immunoreactivity in the renal section of the rat injected with Cu(II) exhibited a low level (Figs. 2D, 3D, and 5G), because Cu administration was carried out only three times. Injected Cu ions were considered to be primarily concentrated in liver; the Cu content in liver was about 10 times higher than that in kidney (data not shown).

We observed a large concentration of Cu-MT in lysosome-like organelles in the PCT cells of the rats by microscopic studies (Fig. 5, A–F). Evering et al. (10), Okabe et al. (20), and Suzuki-Kurasaki et al. (29) reported that Cu-MT was incorporated into the lysosomes in the Cu-loaded rat kidney, LEC rat kidney, and macular mouse kidney, respectively. Several reports have shown that bound metal ions are released from MT by lysosomal degradation (3, 11, 29, 32). The inducer of newly biosynthesized MT in the cortex was estimated to be Cu ions released from partially degraded Cu-MT in the lysosome-like granules in the cortex (Figs. 4C and 5, A–F). We already support this "lysosomal theory" that the protein is degraded when it is reabsorbed into the PCT system in the kidney as previously described (29). The metals bound to MT are concentrated in the lysosomes 30 min after MT administration (24). We propose a lysosomal cycle of Cu-MT in rat kidney as indicated in Fig. 7. Cu-MT of other organs (in this study administered Cu-MT) was transported into PCT cells in the cortex by the bloodstream and was taken up to the lysosome. The released Cu ions from
Cu-MT in the lysosome were reabsorbed by these cells, especially in the S1 and S2 segments, and acted as an inducer of MT mRNA in the same region, suggesting that Cu-MT is continuously synthesized in this region. Then the newly synthesized Cu-MT could be received by degradation in the lysosome. During the circulation of Cu ions (Fig. 7), the metals in the PCT cells could cause renal injury in the rat. Cd-MT in cells causes renal damage due to free Cd ions released from Cd-MT by the lysosomal degradation or oxidation of the protein (7). The physiological significance of this result is that PCT cells are considered to be the primary site of the nephrotoxicity caused by heavy metals, such as Cd (7) and Cu (29). The lysosomal cycle described here does not contradict the theory of the lysosomal system for Cd-MT reported by Nordberg and Nordberg (18). Although in samples of renal tissue from uninephrectomized and sham-operated rats, the rates of transcription and cDNA in LEC rat and normal rat kidneys (unpublished observations).

REFERENCES


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