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依頼論文

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【要　　旨】

本研究ではウィルソン病患者ならびにLECラットにおける尿中の銅量および銅の性質を検討した。ラット由来の全ての尿試料と、幾つかの患者から提供された尿試料でCu (II) のESRシグナルが観測されたが、健常ボランティアから採取した試料からはこのシグナルが観測されなかった。Cu (II) シグナル強度は銅濃度にほぼ比例した。LECラットでは10週、17週、21週と週齢が進むのに対応してCu (II) シグナル強度、総アミノ酸量が増加した。アミノ酸量は常に銅量の100倍以上であり、これに対し総グルタチオン量は僅かであった。尿試料の測定でもCu (II) シグナル強度ならびに水酸ラジカル生成能はICP-MSにより決定した銅濃度にほぼ比例した。尿試料を分子量限界5000程度のろ過にかけてもESRの分析結果は変わらなかった。尿試料の分析で観測されたESRシグナルは、高分子には結合せず、酸化還元活性のある状態の銅の出現に由来し、シグナル強度は肝ないし腎からの銅のオーバーフローの度合いを示すだろう。

キーワード　銅、ESR、LECラット、活性酸素種、尿、ウィルソン病

Introduction

Copper is one of the transition elements that can catalyze the production of reactive oxygen species (ROS) in what is called the Fenton reaction; regulation of copper levels in the body is, therefore, important[1]. Copper is absorbed by amino acids, circulated mainly as ceruloplasmin (Cp) or with albumin, stored with metallothioneins (MTs), and excreted mainly into the bile or urine, saliva and hair[2]. Copper absorption competes with Zn^{2+} [3] or any excess amino acids[4] and absorption is inhibited by MTs[5, 6].

The Long-Evans Cinnamon rat (LEC rat) provides a model of Wilson's disease (WD) caused by mutations in the ATP7b gene[7, 8], which result in abnormal accumulation of copper in tissues such as the liver or kidneys, in an age-dependent manner. In the LEC rat, acute hepatitis develops spontaneously about 4 months after birth, with clinical features similar to those seen in human fulminant hepatitis, sometimes a feature of WD. This hepatitis can be prevented by treatment with copper-chelating agents such as D-penicillamine (D-Pc), which facilitate the excretion of copper. Zn^{2+}-treatment is also thought to be effective because of its effect upon the induction of MTs. MTs in rat livers sequester Cu to prevent Cu-dependent oxidative stress and scavenge oxygen radicals[9].

We investigated changes in the amount and species of copper using LEC rat livers before and after the onset of hepatitis[10, 11]. However, methods used to measure liver-copper levels inflict heavy stress on model animals and even more so on patients. Urine, however, is non-invasively collectable; the present study, therefore, was undertaken to determine copper levels and copper species in urine from WD patients and LEC rats.

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Materials and Methods

***Collection of Urine from Rats*[12]**

LEC rats (2 male and 2 female) were fed a laboratory diet and tap water *ad libitum*. At 10, 17 and 21 weeks after birth (described briefly as 10-week-old, 17-week-old and 21-week-old, respectively), urine samples of over 5 milliliters were collected into tubes continuously over a 12-hour period; these were screened and cooled on ice. Samples were frozen and stored at －80℃ until use. LEC rats' conditions were classified as: 10-week-old, before jaundice; 17-week-old, just before jaundice; 21-week-old, after the onset of jaundice. Wistar rats (2 male and 2 female) at 10 weeks after birth were treated as the control.

Urine Samples from WD Patients

Urine samples from WD patients just after diagnosis were withdrawn as spot urine in the following way: from patient A, 5 months before (n=2) and after (n=29) D-Pc treatment; from patient B, 4 days before and after D-Pc treatment; from patient C, D and E, one sample each before D-Pc treatment. Urine samples from healthy child and adult volunteers were also collected as spot urine.

Holo-Cp was detected by an enzyme-linked immunosorbent assay (ELISA) kit with an anti-human Cp monoclonal antibody (Nissho Inc., Japan).

Copper Analysis

Copper concentrations were determined with an ICP-MS spectrometer, SPS4500 (Yokogawa Analytical Systems, Japan). Statistical analysis was carried out using a Microsoft Excel add-in-tool.

Cu(II) in urine was detected directly with an ESR spectrometer, JEOL JES-TE300 with 100 kHz modulation at room temperature with a quartz flat cell. Both intact samples and those after ultrafiltration with Millipore ULTRAFREE® -MC (5,000 NMWL filter unit) were analyzed. The presence of several kinds of ligands for copper in urine was examined[13, 14]. The calibration curve for the determination of copper concentrations was made with a copper-glutamate (Cu-Glu) mixture (0-0.2 mM $CuCl_2$ and 3-20 mM glutamate in 10 mM phosphate buffer, pH 7.0) as a copper-amino acids complex standard. A Mn(II) marker as reference was also used to calculate the parameters g_0 and A_0, and to estimate the ESR signal intensity. The instrumental conditions were: frequency, 9.42 GHz; power, 8.0 mW; modulation amplitude, 1 mT; time constant, 10 sec; scan rate, 6.7 mT/min.

Cu(II) was also analyzed with an ion meter equipped with a cupric electrode, Model 720A (Thermo Orion). Calibration was performed with 25 ml of 1, 10 and 100 ,,M $Cu(NO_3)$ with stirring.

Amino Acids

Total concentrations and compositions of amino acids were determined with an autoanalyzer, Pico-Tag/AccQ-Tag amino acids analysis system (Waters) equipped with Pico-Tag Column (3.9 × 300 mm) and UV-detector for PITC-derivatives (254 nm).

Glutathione

Total glutathione (GSH + GSSG) concentrations were determined as fluorescent derivative with *o*-phthalaldehyde[15]. Aliquots (10 or 50 μl) of samples were added to 2ml of 0.1 M NaOH then reacted with 20 μl of *o*-phthalaldehyde solution (10 mg/ml ethanol) and left for 15-30 min. Fluorescence (Ex. 350 nm, Em. 420 nm) was measured with a Shimadzu RF-5300 spectrofluorometer.

Detection of reduced glutathione (GSH) was attempted using Ellman's reagent, 5,5'-dithiobis -2-nitrobenzoic acid (DTNB).

Ascorbate, Protein, Glucose and pH

The presence of ascorbic acid, protein and glucose and pH were measured using test paper, Pretest 4bII (Wako Pure Chemical Industries, Ltd., Japan); the former three parameters reflect DCIP reduction, tetrabromophenol blue binding and glucose oxidase-peroxidase-*o*-tolidine coloration, respectively.

***Cp Activity*[16]**

Cp oxidizes *o*-dianisidine at pH 5.5, and products appear purple (molar absorption coefficient at 540 nm is 9.6 $mM^{-1}cm^{-1}$) in strong acid (final 6 M H_2SO_4). Aliquots (25 μl) of samples were added to 375 μl of 0.1 M sodium acetate buffer (pH 5.0) and incubated for 5 min at 30℃, then reacted with 0.1 ml of *o*-dianisidine solution (0.25%) and left for 1 min (not reacted) or 15-30 min (reacted). Reactions were stopped and color developed with 1.0 ml of 9 M H_2SO_4. Increase of absorbance at 540 nm was

measured with a Shimadzu MPS-2000 spectrophotometer.

ESR-Spin Trapping of Oxygen Radicals with DMPO

Reactions of the urine or copper-complex with H_2O_2 or D-Pc were observed by the ESR spin-trapping method using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as the spin trap[17]. The reaction mixture (130 μl) contained 65 μl of urine or Cu-Glu (0.2 mM $CuCl_2$ and 3 mM Glu in 10 mM phosphate buffer, pH 7.0), 100 mM DMPO and 1 mM trientine, $HgCl_2$, H_2O_2 or D-Pc, respectively. DMPO-adducts were observed at room temperature with a quartz flat cell. A Mn(II) marker as reference was used to calculate the ESR signal intensity. The instrumental conditions were: frequency, 9.42 GHz; power, 2.0 mW; modulation amplitude, 0.1 mT; time constant, 0.3 sec; scan rate, 2.5 mT/min.

Results

Copper in urine

Copper excretion into the urine analyzed by ICP-MS increased with the age of the LEC rats as expected. The ESR signal due to Cu(II) was observed in all urine samples from the LEC rats (Fig. 1Ab-d) and Wistar rats (Fig. 1Aa). In all urine samples, pH was neutral or weakly alkaline. The intensity of the Cu(II) signal in the urine increased in an age-dependant manner in the case of the LEC rats. Fig1Ac indicates that the LEC rats had already excreted large amounts of copper just before the onset of jaundice.

The ESR signal intensity highly correlates with the ESR signal height under conditions where the special ligand is dominant[10, 11], which makes the signal line width constant. The intensity of the Cu(II) signal in the urine was proportional (r^2=0.93) to the concentrations of copper determined with ICP-MS (Fig. 2A): 21-week-old, 156±56 ,,M, n=2 (male only); 17-week-old, 48±30 ,,M, n=4; 10-week-old, 14±6 ,,M, n=4).

Compared to standard Cu(II) solutions, these ESR spectra of urine from the rats resemble that of the copper-amino acid complex, at least the 1N3O, 2N2O and/or 3N1O chelating pattern (Table I)[18]; although measurements of the ESR signal at room temperature used to result in less variation in g_0 value and in a narrower A_0 without weak ligands like AcOH, HCO_3^- etc.[14]. The relative affinity of amino acids and chelating agents for Cu are shown in Fig. 3. In order to obtain maximum intensity, the ligand-metal ratio had to be over 10. There was no significant differences in the ESR spectra of the copper complexes in the following amino acids: Ala, Glu, Gly, His and Lys, while it was noted that N-α-acetylation of Lys changed the ESR spectra of the Cu-Lys complex (data not shown).

An identical ESR signal due to Cu(II) was observed in several urine samples from patient A (Fig. 1B) and patient B (data not shown), in contrast with the data from healthy child and adult volunteers' samples: Cu in their urine were under the detection limit with both ICP-MS and ESR. In these patients, the intensity of the Cu(II) ESR signal increased after D-Pc treatment. In the case of patient A, the extensive excretion of Cu continued 2-3 days after a single dose of D-Pc treatment. The intensity of the Cu(II) signal in the urine was proportional to the concentrations of copper determined with ICP-MS to a certain extent (Fig. 2B). However, no signal was detected when Cu concentrations were less than 20 μM and there was a difference in the ratio of ESR signal intensity/ ICP-MS value ($p<0.05$) between Fig. 2A and Fig. 2B (n=30, r^2=0.34). No effects of the Hg-treatment and H_2O_2-treatment of the urine samples were observed. Ultrafiltration (Mr limit: ca.5,000) of the urine had no effect upon either the intensity or the spectrum of the Cu(II) signal. These results indicate that the urine samples contained neither MTs nor Cu(I)[10, 11] but the existence of oligopeptides (whose complex with copper resembles Cu(II)-amino acids), the interference effect of carbonate or carboxylic acids (which reduce the ESR signal height), and the presence of some ESR-silent species of up to 20 ,,M of Cu could not be denied.

Amino acids and protein concentrations in urine

The concentrations of total amino acids in the urine increased with age in the case of the LEC rat. In all cases, the concentrations of total amino acids were 2 orders of magnitude higher than the concentrations of copper. A marked increase in amino acid concentrations was observed at the onset of hepatitis, whereas no specific amino acids (e.g. His could be expected), that correlated with copper concentrations, were observed (Table II). Protein concen-

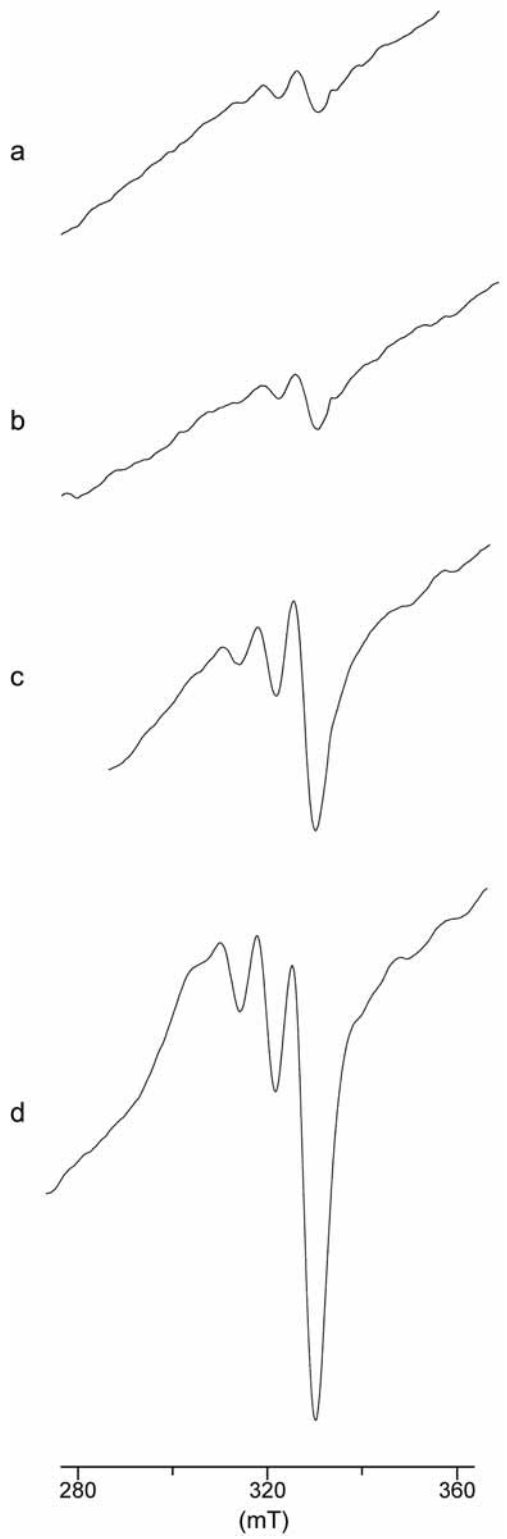

Fig. 1A

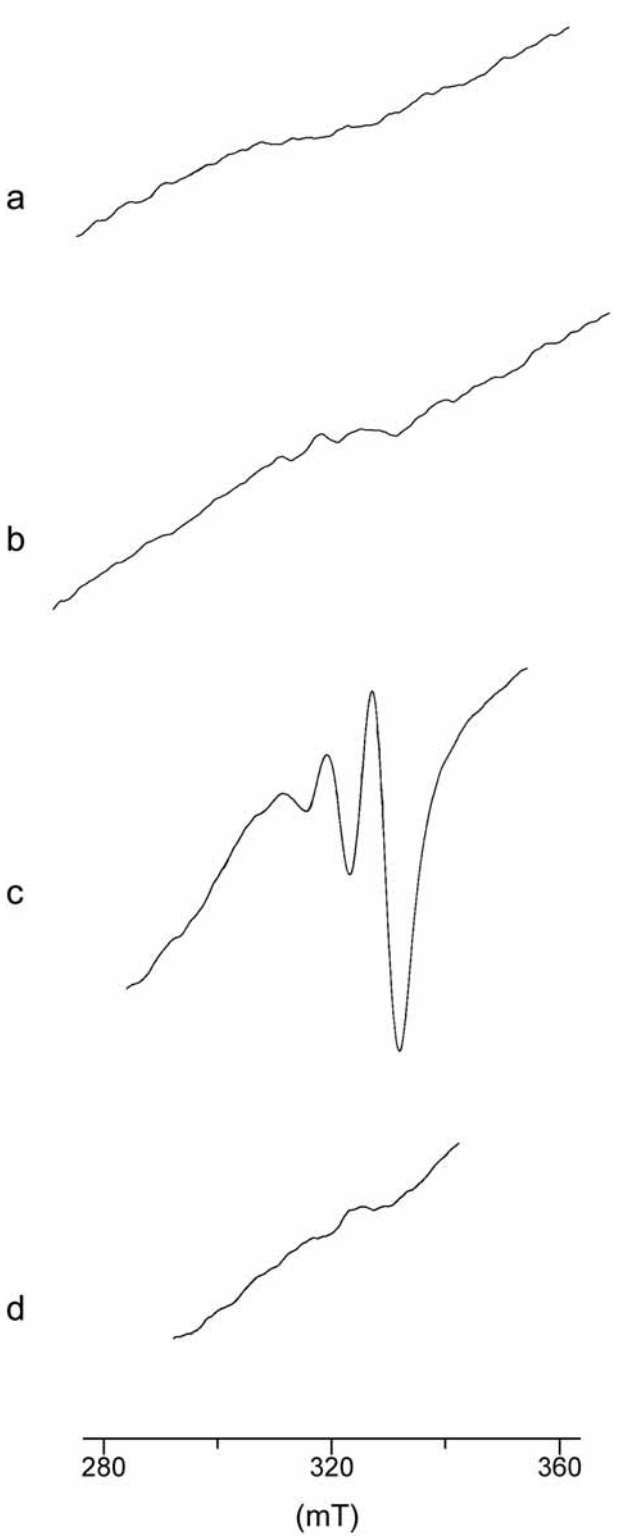

Fig. 1B

Fig. 1. ESR spectra due to Cu(II).

A, ESR spectra of urine from 10-week-old Wistar (a), 10-week-old LEC (b), 17-week-old LEC (c) and 21-week-old LEC (d) male rats.

B, ESR spectra of urine from patient A before (a) and 2 (b), 6 (c) and 17 weeks (d) after D-Pc treatment.

The experimental conditions are described in Materials and Methods.

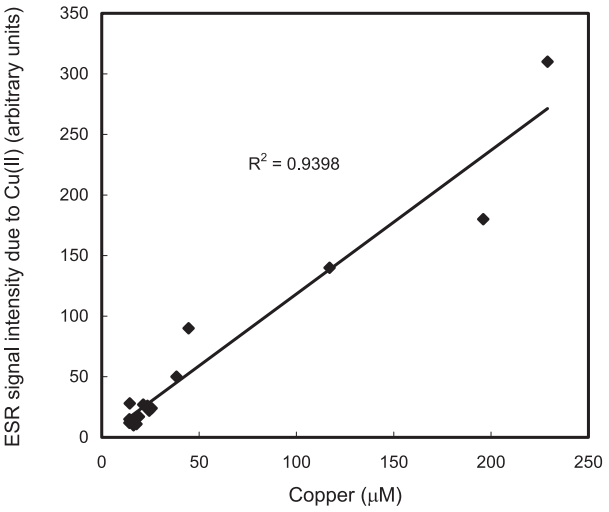

Fig. 2A

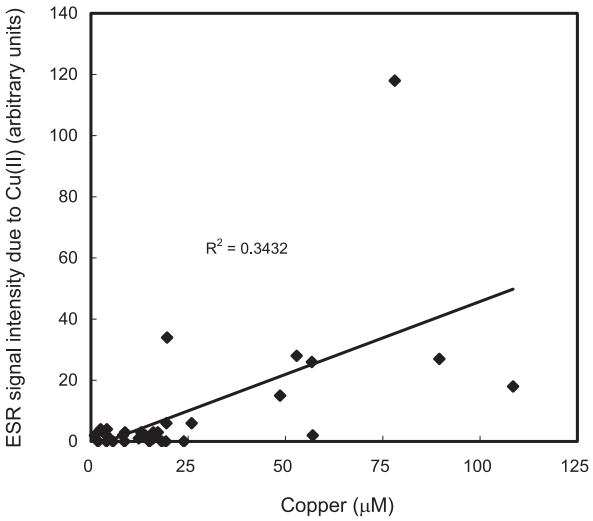

Fig. 2B

Fig. 2. Correlation between concentrations of copper and ESR signal intensity due to Cu(II) in urine from rats (A) and from WD patient (B). Statistical analysis was carried out using Microsoft Excel add-in-tool.

Table I. ESR parameters of Cu(II).

The parameters g_0 and A_0 were calculated by measuring Mn(II) marker as reference.

Ligand	Ligand/Cu	g_0	A_0 (mT)
Gly	10	2.05	7.2
trientine	1.1	2.01	8.3
GSSG	2	2.05	7.5
D-Pc dimer	1.2 -6	2.06	7.5
GSH	＞3		
D-Pc	＞3		
SIGMA Cp		2.05	7.2
LEC rats' urine		2.04	6.9 -8.5
Urine from patients		2.04	6.9-8.3

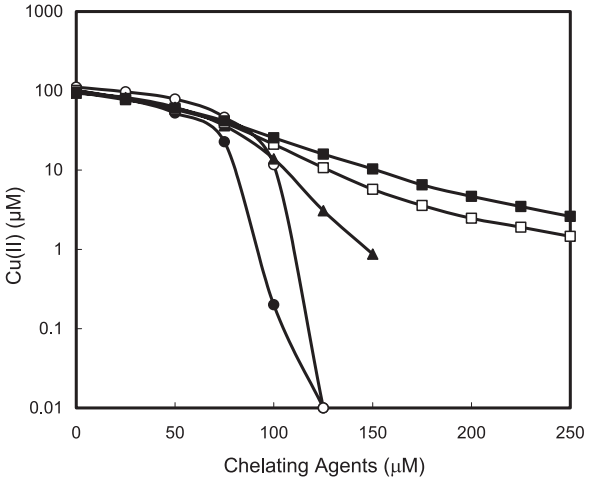

Fig. 3. Copper binding to chelating agents. Reaction mixture contained 0.1 mM $CuCl_2$ in 5 mM HEPES (pH 7.0). Copper concentrations were measured in the absence or presence of varied concentrations of EDTA (●), GSSG (○), His (▲), Glu (□)or Ala (■).

trations were under the detection limit.

Similarly, no specific amino acids, which correlated with copper concentrations, were found in the case of WD patients. The concentrations of total amino acids were 2-3 orders of magnitude higher than the concentrations of copper. However, amino acid concentrations in the urine from patient A increased after D-Pc treatment and were maintained after the decrease in Cu-levels (data not shown).

Glutathione in urine

The concentration of total glutathione (GSH + GSSG) in the urine increased with aging in the LEC rats (Table III). No DTNB-reducing activity in the urine samples was observed. The results indicate that glutathione increased with the increasing concentration of Cu in the urine samples, however, no high correlation between glutathione levels and the concentration of Cu was observed.

In the case of WD patients, glutathione concentrations were also low (299 μM±136 μM, n=17) before and after D-Pc treatment, as were Cu concentrations, and no reducing

Table II. Content of amino acids in urine from rats (n=1) and from patient A.

Abbreviations are: 21WCM, 21-week-old male LEC rat; 17WCM, 17-week-old male LEC rat; 10WCM, 10-week-old male LEC rat; 10WWisM, 10-week-old male Wistar rat.

Amino acids	Concentrations (mM)				
	21WCM	17WCM	10WCM	10WWisM	Patient A (SD, n=13)
phospho-Ser	1.16	0.00	0.00	0.08	0.17 (0.24)
Asp	0.08	0.01	0.17	0.36	0.03 (0.03)
Glu	6.70	0.04	0.23	0.78	0.18 (0.13)
aminoadipic acid	0.24	0.40	0.03	0.24	0.05 (0.02)
hydroxy-Pro	0.00	0.00	0.00	0.00	nd
phenethylamine	0.36	0.06	0.42	0.68	0.09 (0.06)
Ser	4.30	0.21	0.06	0.11	0.10 (0.12)
Gly	3.83	1.84	0.15	0.37	0.90 (0.48)
Gln	14.77	0.00	0.01	0.10	0.25 (0.14)
beta-Ala	0.42	0.00	0.03	0.23	0.06 (0.03)
sarcosine	0.26	0.11	0.20	0.38	0.06 (0.04)
taurine	15.57	0.25	0.37	0.94	1.09 (0.52)
His	2.08	0.08	0.03	0.08	0.50 (0.27)
gamma- aminobutylic acid	0.02	0.02	0.01	0.04	0.03 (0.01)
citrulline	2.88	0.05	0.03	0.06	0.45 (0.30)
Thr	10.74	15.24	0.05	0.10	0.09 (0.06)
Ala	2.34	0.00	0.22	0.45	0.23 (0.11)
beta-aminoisobutylic acid	0.19	0.20	0.03	0.05	2.04 (0.99)
carnosine	0.00	0.14	0.00	0.00	0.12 (0.10)
Arg	0.31	0.05	0.08	0.09	0.01 (0.01)
Pro	0.84	0.14	0.16	0.64	0.03 (0.02)
1-methyl-His	0.92	0.06	0.02	0.00	0.38 (0.40)
Asn	0.00	0.02	0.00	0.00	0.25 (0.10)
3-methyl-His	0.26	0.00	0.01	0.03	0.10 (0.13)
ethanolamine	0.52	0.00	0.21	0.31	0.69 (0.38)
alpha-aminobutylic acid	0.37	0.34	0.02	0.05	0.05 (0.05)
Tyr	1.26	0.02	0.04	0.05	0.09 (0.05)
Val	0.73	0.04	0.05	0.06	0.03 (0.02)
Met	1.15	6.66	0.06	0.14	0.10 (0.06)
cysteic acid	0.17	0.03	0.09	0.11	0.08 (0.01)
cystine	1.47	0.07	0.09	0.25	0.08 (0.05)
Ile	0.45	0.01	0.04	0.05	0.06 (0.03)
Leu	0.72	0.06	0.07	0.06	1.34 (0.87)
hydroxy-Lys 1	0.04	0.20	0.01	0.03	0.02 (0.01)
hydroxy-Lys 2	0.02	0.03	0.00	0.02	0.07 (0.03)
Phe	0.39	0.13	0.06	0.05	0.04 (0.02)
Trp	0.07	0.00	0.04	0.07	1.32 (0.99)
ornithine	0.15	0.00	0.00	0.00	0.05 (0.02)
Lys	0.36	0.00	0.05	0.07	0.04 (0.02)

Table III. Contents of glutathione and amino acids in urine from rats.

Abbreviations are the same as shown in Table II.

Subjects	Concentrations (mM)			
	21WCM	17WCM	10WCM	10WWisM
Total copper	0.196	0.044	0.014	0.014
GSH + GSSG	0.56	0.23	0.09	0.18
Total amino acids	76.1	26.5	3.18	7.09

activity of DTNB was observed. The correlation between total glutathione levels and the concentration of Cu was also lower.

Ascorbic acid and Cp activity in urine

Fresh urine samples before jaundice contained millimolar concentrations of ascorbic acid while only those urine samples from 21-week-old LEC rat had slightly reduced DCIP after storage at －80℃. A urine sample from a 21-week-old LEC rat contained detectable amounts of glucose. None of the urine samples oxidized *o*-dianisidine.

Concentrations of Cp in human urine samples analyzed by ELISA were around 1 nM. Thus, the ratio of Cp to copper was too low to find any correlation between the concentrations of Cp and copper.

Redox activity of Cu in urine

To further confirm whether copper in urine is not bound tightly, the effects of chelating agents on urine were studied (Fig. 4A). The addition of trientine into urine immediately changed the Cu(II) ESR spectra to that of a Cu(II)-trientine complex. D-Pc and GSH eliminated the Cu(II) signal in urine. However, the effect of D-Pc was temporary and the original spectra reappeared at 30 min after the addition of 1 mM D-Pc. Disulfide of D-Pc or GSSG had no effect upon the Cu(II) ESR spectra in the urine. Similar effects of chelating agents upon the Cu-Glu mixture (1:15) were also observed (Fig. 4Bb). The ESR spectra of urine from patients also resembled that of the copper-amino acid complex (e.g. Cu-Gly) although slightly differ from that of the urine of the rats.

ROS produced by samples were measured as DMPO-OH. Both urine (from a 21-week-old LEC rat, Cu=0.2 mM) and the Cu-Glu mixture (or Cu-His, Cu-Gly, $CuCl_2$) produced a weak DMPO-OH signal in the reaction with H_2O_2 (Fig. 5Aa and Ba). On the other hand, the D-Pc-induced DMPO-OH signal developed in the presence of urine or the Cu-Glu mixture (or Cu-His, Cu-Gly, $CuCl_2$) (Fig. 5Ab and Bb), however, the reaction was finished completely at 10 min after the reaction had started. GSH-induced DMPO-OH production was not observed. Similarly, neither trientine-induced nor $HgCl_2$-induced DMPO-OH production was observed.

Similar observations were obtained when urine from a 21-week-old LEC rat was replaced by Cu-containing urine from patient A in the experiment system mentioned above.

Discussion

We showed that Cu(II) was detectable as an ESR signal in all urine samples from rats and some urine samples from WD patients. In some cases, an ascorbate-like signal was also observed simultaneously, although no DCIP-reducing activity was detected. The concentration of ascorbate in human urine would, naturally, vary with the diet. However, even if we take this into consideration, ascorbic acid would not disrupt the ESR signal intensity due to Cu(II) or the determination of copper concentrations. The present finding are completely different from our previous report on liver-copper status, where we found that it was almost always bound to MTs and GSH and thus reduced[11]: copper in the liver is redox-inactive while copper in the urine seems not to be.

Observed ESR spectra indicate that a plausible candidate for the ligand of copper in urine could be amino acids, at least those without macromolecules. The relative affinity of amino acids and chelating agents for Cu(II) are shown in Fig. 3. The affinity of amino acids for copper is slightly lower than chelating agents like EDTA (logK values at neutral pH are 6 for Gly etc., 9 for His and 18 for EDTA, respectively). Titration experiments with ESR revealed that the ratio of Glu/Cu required is over 3 for maximal intensity of the signal, whilst the ratio of trinetine/Cu is 1. The ratio of amino acids to copper in the urine was about 100, hence enough to form a complete copper-amino acid complex. It is possible that no competing ligands in the urine exist. However, diabetic urine contains high amounts of protease, which would result in peptiduria and aminoaciduria[19]. If present in the urine, peptide bonds in polypeptides, such as thioneins, would not be better ligands than the free alpha amino-and carboxyl-groups in the amino acids. The concentrations of glutathione and protein were rather found to be lower than the amino acids. The addition of GSSG (at physiological concentration) into the urine did not change the ESR spectra of urine. The results agree with the relative affinity of amino acids and chelating agents for copper (Fig. 3). Reaction of the Cu(II)-amino acid complex with GSH or

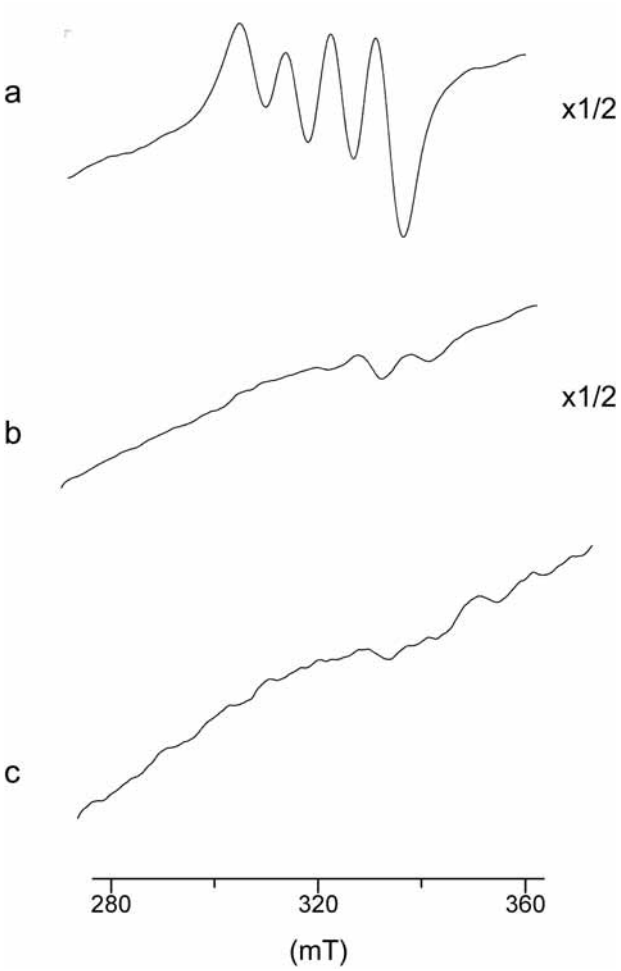

Fig. 4A

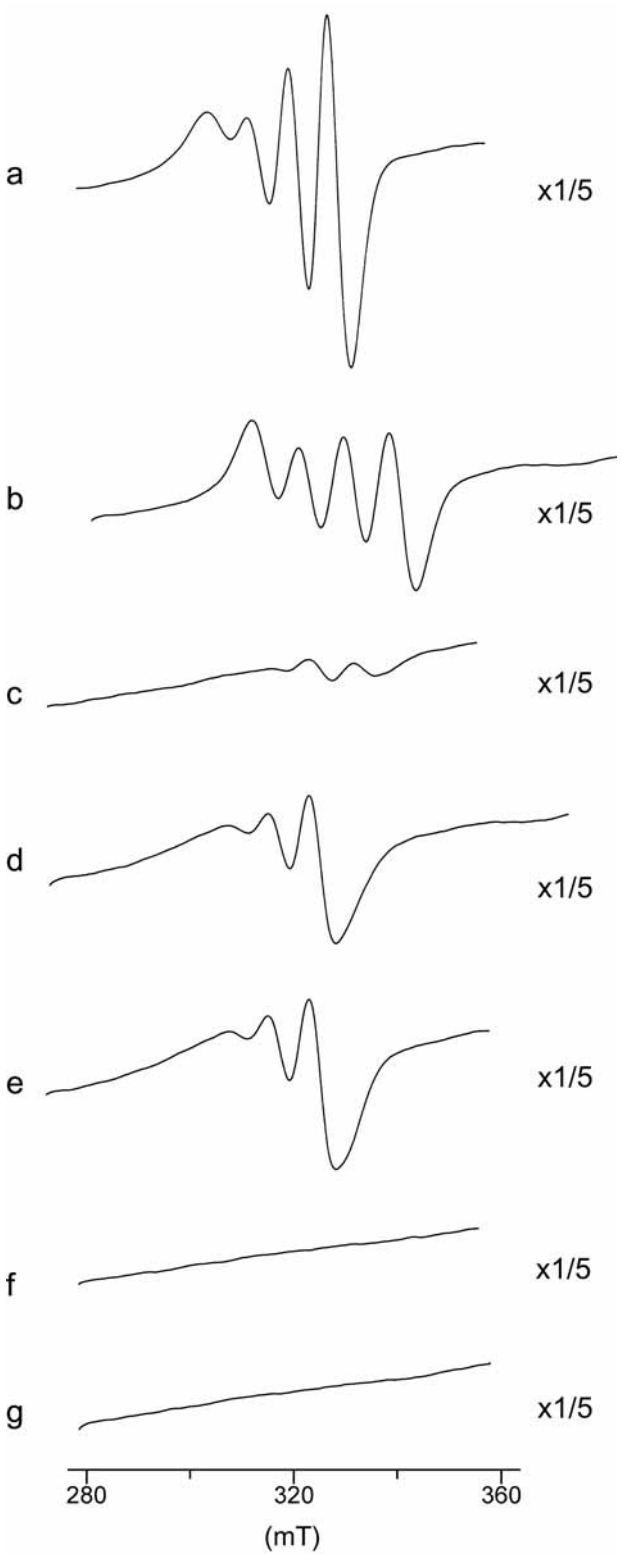

Fig. 4B

Fig. 4. Effects of chelating agents upon ESR spectra due to Cu(II).

A, ESR spectra of urine from 21-week-old LEC rats. Spectra of urine samples in the presence of 1 mM trientine (a), 1 mM D-Pc (b) or 1 mM GSH (c) were recorded as Fig. 1Ad.

B, ESR spectra of Cu-Glu complex: a, 0.2 mM $CuCl_2$ and 3 mM Glu in 10 mM phosphate buffer (pH 7.0); b, (a) + 1 mM trientine; c, d, (a) + 1 mM D-Pc (1 min, 30 min after mixing); e, (a) + 1 mM D-Pc disulfide; f, g, (a) + 1 mM GSH (1 min, 30 min after mixing).

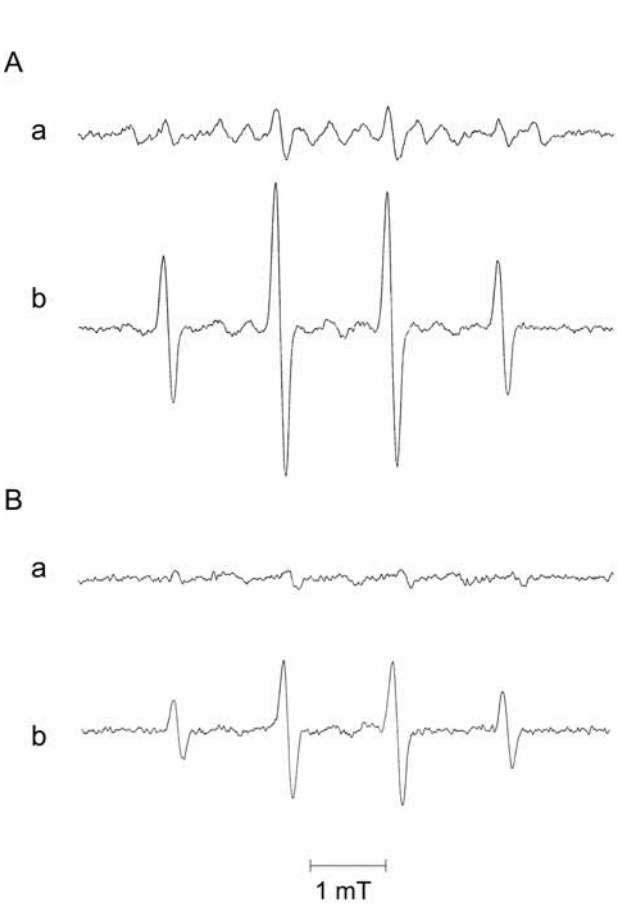

Fig. 5. Hydroxyl radical formation in the reaction of H_2O_2 (a) or D-Pc (b) with urine (A) or Cu-Glu complex (B).
Spectra were recorded immediately after mixing the urine sample from a 21-week-old LEC rat (containing 0.1 mM copper as the final concentration) or a Cu-Glu complex with DMPO solution. Details of these ESR-spin trapping experiments are described in Materials and Methods.

D-Pc was followed by the ESR method. A complete loss of the Cu(II)-amino acid signal required a 3 fold molar excess of GSH and a 10 fold molar excess of D-Pc to Cu(II). One-electron oxidation of thiol compounds by Cu(II) was confirmed by the formation of DMPO-thiyl radical adduct. It has been documented that the D-Pc thiyl radical is more unstable than the GSH thiyl radical under aerobic conditions[20]. Cu(II)-amino acid and -adenine complexes are redox active[21-23]. With the addition of ascorbic acid into the Cu-Glu mixture (or Cu-His, Cu-Gly, $CuCl_2$), ascorbate radicals signals were observed (unpublished data); compatible with the finding that no substantial amounts of ascorbate are found in LEC rat urine. Hydroxyl radicals were detected during the reduction of the Cu-Glu mixture with H_2O_2 or D-Pc (Fig. 5). Cu-His instead of Cu-Glu also gave the same results. Furthermore, urine from the rats or WD patients produced hydroxyl radicals in the reaction with H_2O_2 in those cases that contained copper. Again, these results suggest that copper in urine might be redox active.

The proline effect on liver failure of the LEC rat has already been explained[24]. In general, amino acids most abundant in human urine are Gly, His, Lys and Gln. Our results indicate that, when specific pattern of amino acids was not observed, no special event such as degradation of collagen or MTs had taken place with the onset of hepatitis. With respect to removing the copper, there were no significant differences among amino acids on chelating copper at a neutral pH because the ESR spectra of the copper-amino acid complexes were not affected by the molecule side chains but only by N-α-acetylation.

LEC rats share a common feature with patients of WD: a deficiency in ATPase 7B, a copper transporter. In fact, WD is often initially diagnosed as hepatitis. On the other hand, monitoring urine copper, serum holo-Cp, Kayser-Fleischer ring, etc. may support the diagnosis of the disease. One of the characteristics of WD is an increase of copper excretion into the urine (>1.6 μmol/day)[25] and the ESR-detectable level of copper is over 10 μM. In the present study, ESR-detectable copper was observed only during D-Pc treatment; in particular, extensive excretion of Cu was observed between 4 and 10 weeks after the beginning of treatment. This observation matches with the mention in other previous reports[26]. Small, basal excretion of Cu up to 20 „M seemed to occur and the level could be varied by diet. Moreover, inpatients had meal restrictions since they were hospitalized. Thus, the effectiveness of the diagnosis at an early stage using ESR would not be high in WD. Genetic diagnosis, combined with mass screening using a urine Cp test[27, 28], might be the only definite way at present to detect the disease before onset.

Renal failure in LEC rats and WD patients may occur because of heavy metal toxicity on the proximal tubule, as

proposed by Nomiyama K *et al.*[29], inversely urinary copper may be the result of oxidative stress in the case of type 2 diabetes mellitus[30,31]. The abnormal accumulation of copper with age in LEC rat tissue is associated with an increase of redox active copper, causing oxidative stress[9, 32]. Copper-and iron-induced Fenton reactions, which lead to the formation of hydroxyl radicals, may play an important role in the damage to the liver and kidneys. D-Pc could amplify this through production of ROS in the reaction between copper reduced by D-Pc and oxygen, besides encouragement of copper excretion. However, the amounts of both D-Pc and D-Pc dimer in any urine samples were below detection limits by mass-spectrometry analysis (unpublished data) and ESR analysis. Because excretion of Cu-D-Pc complex into the urine could not be proved from our data, the problem of D-Pc (participation upon renal failure and acceleration of Cu excretion) remains unclear.

Absence of Cp activity in the urine has not yet been proven, because measurement was performed after storage at −80℃. Below zero degrees Celsius, inactivation of Cp may occur. Assuming that inactivation does not occur, the copper in urine would be predominantly Cp-free, in contrast with blood in which it is nearly all bound up as Cp. Protein was detected neither in the urine samples from 21-week-old LEC rats nor in the urine samples from WD patients. Attempts to determine urinary MTs[33-37] indicate that the maximum amount of urinary MTs would be several nanomolars, which is insufficient to bind all the copper molecules that exist in urine (at maximum a few micromolars) even if MTs had been extensively inducted by accumulated copper in the WD patients or LEC rats. In this study, the ligand(s) for Cu in the urine samples could not be identified although the majority of Cu is macromolecule-free, loosely bound and redox-active.

In conclusion, Cu(II) was detectable as an ESR signal in all the urine samples from rats and some of the urine samples from WD patients. The observed ESR spectra of urine could be attributed to the emerging of macromolecule-free copper that is redox-active and the ESR signal intensity of urine due to Cu(II) may indicate the degree of Cu-overflow from the liver and/or kidneys.

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Determining the status of copper in the urine of Long-Evans Cinnamon rats and patients with Wilson's disease

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Summary

We attempted to determine copper levels and copper states in the urine of WD patients and LEC rats. ESR signals due to Cu(II) were observed in all urine samples from the LEC and Wistar rats and in some urine samples from the WD patients but not in the urine samples of healthy human volunteers. There was a good correlation between the intensity of the Cu(II) signal and Cu levels. The intensity of the Cu(II) signal and amino acid contents in urine increased with age in the case of LEC rats. In all cases, the concentrations of total amino acids were 2 orders of magnitude higher than the concentrations of copper, and the content of total glutathione was considerably less than that of amino acids. The intensity of the Cu(II) signal in the urine and the amount of hydroxyl radical formation were proportional to the concentrations of copper determined with ICP-MS. Ultrafiltration (Mr limit: ca.5,000) of urine samples did not alter the results of the ESR analysis. Observed ESR spectra of urine may be attributed to the emergence of macromolecule-free, redox-active copper, while the ESR signal intensity in the urine due to Cu(II) may indicate the degree of Cu-overflow from the liver and/or kidneys.

Key words Copper; ESR; Long-Evans Cinnamon rat; Reactive oxygen species; Urine; Wilson's disease

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