Urate Oxidase Activity and Copper Content in the Liver of Macular Mutant Mouse, a Model Animal for Human Congenital Copper Deficiency, Menkes’ Kinky Hair Disease

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Summary The macular mouse is an X-linked recessive inherited mutant and is considered to be a model for human congenital copper deficiency, Menkes’ kinky hair disease. The activity of urate oxidase, which has been believed to be a copper enzyme, and copper content in the liver of the mutant mouse were determined. The oxidase activity was maintained at normal level even though there was very low level of copper present in the liver through days 7 to 14. Copper administration increased the copper content in the liver to the normal level, but did not affect the oxidase activity.

Key Words urate oxidase, macular mutant mouse, Menkes’ kinky hair disease, kinky hair syndrome, X-linked inherited disorder, copper enzyme, copper deficiency

Menkes’ kinky hair disease is an X-linked recessive inherited disorder in which copper metabolism appears to be disturbed (1, 2). Although the precise nature of the basic defect remains unknown, this syndrome is characterized by growth retardation, progressive cerebral degeneration, distinctive facial features, hypopigmentation of hair and skin, convulsions and kinky hair (3, 4). Majority of the patients die at the age of around 3 with respiratory infections (5).

The brindled mouse, which is an X-linked mutant and a well-known model animal for Menkes’ kinky hair disease, has been studied extensively, and these studies have contributed to the basic understanding of the defect in this syndrome (6, 7). Macular mouse discovered in Japan (8) is also an X-linked recessive inherited mutant and shown to be similar to the brindled mouse in the pattern of copper accumulation or deficiency in tissues (9). Since most features in the disorder of these mutant mice are explicable on the basis of impaired function of
copper-dependent enzymes, several copper-containing enzymes have been studied in tissues or cells of the mutant mice, e.g. lysyl oxidase (10), cytochrome c oxidase (11,12), superoxide dismutase (11) and tyrosinase (13).

Urate oxidase [urate:oxygen oxidoreductase, EC 1.7.3.3] is a peroxisomal enzyme that catalyzes the oxidation of uric acid to allantoin in most mammals, and has been believed to be a copper enzyme, since the purified enzyme from porcine liver was reported to contain copper (14). In humans and certain other primates, however, the enzyme has been lost by a sudden mutational event (15). Although many studies on copper-containing enzymes have been carried out, no reports have been available on urate oxidase in the mutant mice.

This paper reports that the urate oxidase activity in the liver of macular mice is almost the same as that of the normal mice, in spite of copper deficiency in the liver of the mutant mice.

MATERIALS AND METHODS

Animals. The mutant mice have been propagated by mating heterozygous females (M1/+) with normal males (+/y) and maintained in an animal house with constant temperature (22°C) and a 12-h light/12-h dark cycle. Tap water and a commercial stock diet (Japan CLEA CE-2) were provided ad libitum. Hemizygous male mice (M1/y) were used as the experimental animals and their normal littersmates (+/y) as the controls. The mice were weighed and sacrificed on days 7, 10 and 14 postpartum by cervical dislocation. The livers were removed immediately, weighed and frozen at −80°C until required for biochemical analysis.

Another group of mice was injected intraperitoneally with 10, 20 and 20 μg of cupric chloride on days 4, 6 and 8, respectively, as previously described by Kawasaki et al. (16), and was sacrificed on day 10. The livers were removed and frozen as described above.

Preparation of samples. Frozen livers were thawed in 20 vol. of cold 0.1 M sodium-pyrophosphate-HCl, pH 8.5, containing 1% Triton X-100, and then homogenized with a Potter-Elvehjem glass-Teflon homogenizer. The homogenate was centrifuged at 105,000×g for 30 min at 4°C. The supernatant was used for the experiments.

Assay of urate oxidase. Urate oxidase activity was assayed by measuring the oxygen consumption with a galvanic oxygen electrode (MB-1000, Iijima Electronics Mfg. Co., Ltd., Gamagori). The reaction was carried out at 37°C in a closed, 1.8 ml cell, which contained 0.1 M sodium pyrophosphate buffer, pH 8.5, 0.14 mM sodium urate as a substrate and the supernatant. The output of the electrode was calibrated by air-saturated distilled water at 37°C.

Determination of copper content. The supernatant (0.1 ml) was lyophilized, and then wet-ashed in 50 μl of concentrated nitric acid at 60°C for 30 min (17). The clear solution of the ashed sample was diluted 4-times with distilled water and the copper content was measured with a Shimadzu atomic absorption spectro-
photometer AA-660, with a graphite furnace atomizer GFA-4A.

_Determination of protein concentration._ Protein concentration was measured by the method of Lowry et al. (18), using bovine serum albumin as a standard.

_Statistical analysis._ The results shown in tables are expressed as means±SD. Statistical significance was analyzed by one- and two-way analyses of variance followed by means of Student’s t-test. Prior to the analyses of variance, equality of variance was checked by Barlett test.

RESULTS

**Body weight, liver weight and liver copper content**

Changes in body weight, liver weight and copper content in the liver of 7- to 14-day-old mice are shown in Table 1. No significant difference in body weight was observed through days 7 to 14 in macular males (Ml/y). Although the body weight of normal littermates (+/y) at 7 days of age was almost the same as that of Ml/y, the former gained more weight than the latter at 10 days of age, and no significant increase was observed after 10 days.

The copper contents per wet weight of the liver in Ml/y was significantly low compared to those in +/y. The most significant difference in the copper content was observed at 7 days of age and +/y contained 6-fold higher than that of Ml/y. The copper content in Ml/y did not change significantly through days 7 to 14; however, that in +/y showed significant decrease. These findings are consistent with the previous report (9). The analyses of variance revealed that significant differences in body weight and copper content were observed throughout the described age, and between the two genotypes and in their interaction.

Liver weights of both Ml/y and +/y at 7 days of age were almost the same.

| Age (day) | Genotype | Body weight (g) | Liver weight (mg) | Copper (µg/g wet weight) |
|-----------|----------|-----------------|-------------------|-------------------------|
| 7         | +/y (4)  | 4.3±1.0         | 144±37            | 14.5±4.7                |
|           | Ml/y (4) | 3.8±1.0         | 121±38            | 2.5±0.8**               |
| 10        | +/y (6)  | 6.2±0.4         | 178±36            | 7.2±3.5                 |
|           | Ml/y (6) | 4.6±1.0**       | 129±34*           | 2.2±1.4**               |
| 14        | +/y (4)  | 6.3±0.9         | 199±37            | 6.2±3.4                 |
|           | Ml/y (4) | 3.7±0.6**       | 91±10**           | 2.7±1.3                 |

Numbers in parentheses refer to number of animals used, values represent mean±SD, and * and ** denote significant difference at the 5% and 1% probability levels, respectively, compared to corresponding control group. Age was counted from 1 at the day of birth.

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After 7 days, liver weights in +/y increased gradually, whereas those in Ml/y decreased gradually and were about half of those in +/y at 14 days of age. Likewise, significant difference between the two genotypes was observed by the analyses of variance.

**Urate oxidase activity**

Changes in urate oxidase activities per protein and per liver weight shown in Table 2 revealed no significant difference between macular males and normal littermates. The total activities in Ml/y were significantly lower than those in +/y throughout days 7 to 14, but this may probably be due to a decrease in the liver weight of the mutant (Table 1).

**Effect of copper treatment**

The effect of copper treatment on macular males and normal littermates is shown in Tables 3 and 4. Since significant difference in copper contents of the treated groups was observed by Bartlett test due to their large deviation, the logarithms of the values were used for the statistical analyses.

Copper treatment did not significantly affect the body weight and liver weight at 10 days of both Ml/y and +/y. However, the copper contents in the liver markedly increased in both Ml/y and +/y. The copper contents in the treated Ml/y reached a level comparable to that in untreated +/y at 10 days of age (Table 3). Significant difference was observed in the interaction between copper treatment and genotypes, suggesting that the effect of the treatment was different in the two genotypes.

In spite of the increase in copper content, no significant change in urate oxidase activities in both Ml/y and +/y was observed by the copper treatment (Table 4).

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**Table 2. Changes in urate oxidase activity with age in macular males (Ml/y) and normal littermates (+/y).**

| Age (day) | Genotype | Activity per protein (mU/mg) | Activity per liver weight (mU/mg) | Total (mU) |
|-----------|----------|-----------------------------|---------------------------------|------------|
| 7         | +/y (4)  | 4.3 ± 1.9                   | 0.51 ± 0.21                     | 75 ± 42    |
|           | Ml/y (4) | 3.1 ± 0.9                   | 0.39 ± 0.13                     | 43 ± 3     |
| 10        | +/y (6)  | 2.3 ± 0.8                   | 0.35 ± 0.14                     | 59 ± 20    |
|           | Ml/y (6) | 1.8 ± 0.3                   | 0.24 ± 0.06                     | 44 ± 28    |
| 14        | +/y (4)  | 2.1 ± 0.3                   | 0.29 ± 0.07                     | 58 ± 23    |
|           | Ml/y (4) | 2.7 ± 0.5                   | 0.41 ± 0.06                     | 37 ± 4     |

Activity is expressed as mU, defined as nmol of O₂ consumed per min. Numbers in parentheses refer to number of animals used, and values represent mean ± SD. No significant difference between the two genotypes was found by the analyses of variance.

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Table 3. Effect of copper treatment on body weight, liver weight and liver copper content at 10 days old macular males (Ml/y) and normal littermates (+/y).

| Copper treatment and genotype | Body weight (g) | Liver weight (mg) | Copper (μg/g wet weight) |
|-------------------------------|----------------|-------------------|-------------------------|
| Untreated +/y (6)             | 6.2±0.4        | 178±36            | 7.2±3.5                 |
| Treated +/y (6)               | 5.7±0.9        | 184±38            | 66.8±49.7*              |
| Untreated Ml/y (6)            | 4.6±1.0        | 129±34            | 2.2±1.4                 |
| Treated Ml/y (6)              | 4.7±0.9        | 152±41            | 12.1±8.4*               |

All mice were sacrificed at 10 days of age, and the copper treatment was carried out as described under MATERIALS AND METHODS. Numbers in parentheses refer to number of animals used, values represent mean ± SD, and * denotes significant difference at the 2% probability level compared to corresponding untreated group, and 1% probability by logarithmic transformation.

Table 4. Effect of copper treatment on urate oxidase activity.

| Copper treatment and genotype | Activity per protein (mU/mg) | Activity per liver weight (mU/mg) | Total (mU) |
|-------------------------------|------------------------------|----------------------------------|------------|
| Untreated +/y (6)             | 2.3±0.8                      | 0.35±0.14                        | 59±20      |
| Treated +/y (6)               | 2.0±0.7                      | 0.27±0.11                        | 50±25      |
| Untreated Ml/y (6)            | 1.8±0.3                      | 0.24±0.06                        | 44±28      |
| Treated Ml/y (6)              | 2.5±0.5                      | 0.33±0.06                        | 50±14      |

Experimental conditions were the same as in Table 3. No significant difference between the two genotypes was found by the analyses of variance.

Table 5. Effect of the addition of CuCl2 on urate oxidase activity.

| Copper treatment and age (day) | Genotype | Activity per protein (mU/mg) |
|--------------------------------|----------|-----------------------------|
| Untrated 7                     | +/y      | 4.3±1.9                     |
|                                | Ml/y     | 3.1±0.9                     |
| Untrated 10                    | +/y      | 2.3±0.8                     |
|                                | Ml/y     | 1.8±0.3                     |
| Untrated 14                    | +/y      | 2.1±0.3                     |
|                                | Ml/y     | 2.7±0.5                     |
| Treated 10                     | +/y      | 2.0±0.7                     |
|                                | Ml/y     | 2.5±0.5                     |

Values represent mean±SD. No significant difference was found by the analyses of variance.
Effect of copper addition to reaction mixture

By the addition of 5 μM cupric chloride to the reaction mixture, the urate oxidase activity showed no significant increase in both macular males and normal littermates regardless of their age or copper treatment (Table 5).

DISCUSSION

Studies on the model mice of Menkes’ kinky hair disease including macular and brindled mice revealed that most symptoms of the disease are explainable by a primary defect of copper transport. The biochemical features of the mutant mice are characterized by low levels of copper content in most tissues except kidney and intestinal mucosa (7,9), and by decrease in activities of copper-containing enzymes, including lysyl oxidase in skin and aorta (10,19,20), cytochrome c oxidase in brain and heart (11,12,21), superoxide dismutase in brain (11,21) and tyrosinase in melanocytes (13). A reduction in central and peripheral synthesis of noradrenaline has also been demonstrated in mutant mice, indicating the decrease in dopamine β-hydroxylase, a copper-containing enzyme (11,22). Copper treatment of the mutant mice overcomes these enzyme activities.

Despite the very low levels of copper present in the liver, these enzyme activities in the liver are maintained at normal level. For example, cytochrome c oxidase activity is reduced in brain and heart tissue from the copper-deficient mutant mouse, but the activity in liver tissue is unaffected (11,12 and our unpublished data).

Urate oxidase is present in the liver but not in the brain (23). Like the other enzymes in the liver, no significant difference in the activity was observed between macular males and normal littermates. The oxidase activity was not affected by the copper treatment either. Possible explanations for these results are as follows: 1) The concentration of copper is low compared to the normal, but this enzyme activity is strictly regulated and preferentially maintained in the liver. 2) The decreased copper is mainly due to the stored one but not to the functional one as proposed previously (11,12). 3) The copper is not a functional metal for the enzyme.

Since Mahler et al. reported that copper is the essential cofactor of urate oxidase from porcine liver (14), the mammalian enzyme has been believed to be a copper-requiring enzyme. However, the enzyme from ox kidney was reported not to contain any heavy metals (24), and the ones from microbials contain iron as the cofactor (25,26). Urate oxidase has been purified from camel liver recently, but the role of copper in the purified enzyme has not been clarified (27). On the basis of these reports and the data presented in this paper, the possibility that copper is not functioning in the enzyme catalysis cannot be ruled out. To confirm the possibility, we tried to purify urate oxidase from the livers of the present normal mice according to the method of Conley and Priest (28). The purified oxidase was almost homogeneous on polyacrylamide gel electrophoresis in the presence of
sodium dodecyl sulfate. The specific activity and the copper content were obtained to be approximately 3.7 U/mg and 0.01 mol/mol of the subunit, respectively. Characterization of the enzyme is now in progress in our laboratory.

In spite of similarities in biochemical and histological features between human disease and model mice, there is a critical difference between the two. In human disease, many trials to improve the symptoms and save the life have been unsuccessful (29,30), whereas the symptoms of the model mice can be significantly improved and the life span can be prolonged by a single administration of copper at around 7 days after birth (31,32). However, the different distribution of urate oxidase in mouse and human seems not to be correlated to the difference in response to the copper treatment, since the activity in the liver of the mutant mice remained the same as the normal one and was not affected by the copper treatment.

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REFERENCES

1) Menkes, J. H., Alter, M., Steigleder, G. K., Weakley, D. R., and Sung, J. H. (1962): A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration. Pediatrics, 29, 764–779.
2) Danks, D. M., Stevens, B. J., Campbell, P. E., Gillespie, J. M., Walker-Smith, J., Blomfield, J., and Turner, B. (1972): Menkes' kinky-hair syndrome. Lancet, I, 1100–1103.
3) Camakaris, J., Phillips, M., Danks, D. M., Brown, R., and Stevenson, T. (1983): Mutations in humans and animals which affect copper metabolism. J. Inherited Metab. Dis., 6 (Suppl. 1), 44–50.
4) Danks, D. M. (1989): Disorders of copper transport, in The Metabolic Basis of Inherited Disease, 6th Ed., Vol. I, ed. by Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., McGraw-Hill, Inc., New York, pp. 1411–1431.
5) Danks, D. M., Campbell, P. E., Stevens, B. J., Mayne, V., and Cartwright, E. (1972): Menke's kinky hair syndrome. An inherited defect in copper absorption with widespread effects. Pediatrics, 50, 188–201.
6) Hunt, D. M. (1974): Primary defect in copper transport underlies mottled mutants in the mouse. Nature, 249, 852–854.
7) Camakaris, J., Mann, J. R., and Danks, D. M. (1979): Copper metabolism in mottled mouse mutants. Copper concentrations in tissues during development. Biochem. J., 180, 597–604.
8) Nishimura, M. (1975): A new mutant mouse, macular (Ml). Jikken Dobutsu (Exp. Anim.), 24, 185–185.
9) Katsura, T., Kawasaki, H., Yamano, T., and Shimada, M. (1988): Copper contents and pathological changes in various organs of macular mouse. Congenital Anom., 28,
10) Royce, P. M., Camakaris, J., and Danks, D. M. (1980): Reduced lysyl oxidase activity in skin fibroblasts from patients with Menkes' syndrome. Biochim. J., 192, 579–586.

11) Hunt, D. M. (1977): Catecholamine biosynthesis and the activity of a number of copper-dependent enzymes in the copper deficient mottled mouse mutants. Comp. Biochem. Physiol., 57C, 79–83.

12) Rezek, D. L., and Moore, C. L. (1986): Depletion of brain mitochondria cytochrome oxidase in the mottled mouse mutant. Exp. Neurol., 91, 640–645.

13) Holstein, T. J., Fung, R. Q., Quevedo, W. C., Jr., and Bieniek, T. C. (1979): Effect of altered copper metabolism induced by mottled alleles and diet on mouse tyrosinase (40662). Proc. Soc. Exp. Biol. Med., 162, 264–268.

14) Mahler, H. R., Hübscher, G., and Baum, H. (1955): Studies on uricase. I. Preparation, purification, and properties of a cuproprotein. J. Biol. Chem., 216, 625–641.

15) Wu, X., Lee, C. C., Muzny, D. M., and Caskey, C. T. (1989): Urate oxidase. Primary structure and evolutionary implications. Proc. Natl. Acad. Sci. U.S.A., 86, 9412–9416.

16) Kawasaki, H., Yamano, T., Iwane, S., and Shimada, M. (1988): Golgi study on macular mutant mouse after copper therapy. Acta Neuropathol., 76, 606–612.

17) Stevens, B. J. (1972): Biological applications of the carbon rod atomizer in atomic absorption spectroscopy. 2. Determination of copper in small samples of tissue. Clin. Chem., 18, 1379–1384.

18) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.

19) Royce, P. M., Camakaris, J., Mann, J. R., and Danks, D. M. (1982): Copper metabolism in mottled mouse mutants. The effect of copper therapy on lysyl oxidase activity in brindled (Mo^{b+}) mice. Biochem. J., 202, 369–371.

20) Phillips, M., Camakaris, J., and Danks, D. M. (1986): Comparisons of copper deficiency states in the murine mutants blotchy and brindled. Changes in copper-dependent enzyme activity in 13-day-old mice. Biochem. J., 238, 177–183.

21) Hoeldtke, R. D., Cavanaugh, S. T., Hughes, J. D., Mattis-Graves, K., Hobnell, E., and Grover, W. D. (1988): Catecholamine metabolism in kinky hair disease. Pediatr. Neurol., 4, 23–26.

22) Motojima, K., and Goto, S. (1990): Characterization of liver-specific expression of rat uricase using monoclonal antibodies and cloned cDNAs. Biochim. Biophys. Acta, 1087, 316–322.

23) Rainbird, R. M., and Atkins, C. A. (1981): Purification and some properties of urate oxidase from nitrogen-fixing nodules of cowpea. Biochim. Biophys. Acta, 659, 132–140.

24) Osman, A. M., Corso, A. D., Ipata, P. L., and Mura, U. (1989): Liver uricase in Camelus dromedarius: Purification and properties. Comp. Biochem. Physiol., 94B,
28) Conley, T. G., and Priest, D. G. (1979): Purification of uricase from mammalian tissue. *Prep. Biochem.*, 9, 197–203.

29) Garnica, A. D., Frias, J. L., and Rennert, O. M. (1977): Menkes kinky hair syndrome: Is it a treatable disorder? *Clin. Genet.*, 11, 154–161.

30) Maehara, M., Ogasawara, N., Mizutani, N., Watanabe, K., and Suzuki, S. (1983): Cytochrome c oxidase deficiency in Menkes kinky hair disease. *Brain Dev.*, 5, 533–540.

31) Hunt, D. M. (1976): A study of copper treatment and tissue copper levels in the murine congenital copper deficiency, mottled. *Life Sci.*, 19, 1913–1920.

32) Mann, J. R., Camakaris, J., Danks, D. M., and Walliczek, E. G. (1979): Copper metabolism in mottled mouse mutants. Copper therapy of brindled (Mo<sup>W</sup>) mice. *Biochem. J.*, 180, 605–612.