Metabolism of Zinc and Copper in the Neonate

(ZINC,COPPER)-THIONEIN IN THE DEVELOPING RAT KIDNEY AND TESTIS*

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The zinc and copper concentration, content, and distribution (total, particulate, cytosol, metallothionein) in rat kidney (2 to 70 days of age) and in rat testis (10 to 70 days of age) have been quantitated. Kidney and testis metallothionein levels and their zinc and copper compositions have been determined in these animals. Total kidney zinc content rises steadily during this period from 2 to 50 µg/pair of kidneys, while total kidney copper content remains low (<2 µg/pair of kidneys) until weaning, when it begins to increase with age to a level of 15 µg/pair of kidneys at 70 days of age. On a concentration basis total kidney zinc and copper remain fairly stable during this period, being about 25 and 5 µg/g of kidney wet weight, respectively.

Metallothionein in rat kidney is a predominantly zinc-containing protein (Zn:Cu ratio > 4) up to 25 days of age. After weaning there is a slight decrease in the zinc content and a linear increase in the copper content of metallothionein until at 70 days of age the protein is a predominantly copper containing protein (Zn:Cu ratio < 0.65). More than half of the increase in copper content of kidney cytosol which occurs after weaning is bound to metallothionein. Possibly, this copper accumulation in rat kidneys and in renal metallothionein is due to a nutritional change from milk to laboratory chow. From 35 to 70 days of age kidneys from female rats have higher total and metallothionein copper concentrations (micrograms/g of kidney) than do male rats of the same age (p < 0.01), but this is due only to the smaller size of female kidneys, because on a content basis (micrograms/pair of kidneys), there are no significant differences in renal total and metallothionein copper between male and female rats of the same age.

In terms of organ content of metallothionein 70-day-old adult rats have higher amounts of metallothionein in their kidneys than in their livers.

From 15 to 50 days of age the concentration of zinc thionein in rat testis is ≥ 3 µg of zinc in thionein/g of testis wet weight with a peak of 5.2 at 35 days. From 35 days on, the content of zinc thionein exceeds 6 µg of zinc in thionein/pair of testes, a quantity of metallothionein greater than the endogenous level of this protein in the liver and kidneys combined of these same animals.

Metallothioneins are low molecular weight (M, = 6000), cysteine-rich, metal-binding proteins, found in eucaryotes, which are involved in zinc and copper homeostasis and in cadmium and mercury detoxification. Many excellent reviews are available on the various aspects of studies of these interesting proteins (1–3).

In a continuing investigation of the function and role of metallothionein in zinc and copper metabolism this laboratory has systematically studied the levels of (zinc,copper)-thionein in various tissues of the neonatal and developing rat. Studies have been completed on the liver (4), the gastrointestinal tract (5), the kidneys (this study), and the testes (this study). Our working hypothesis has been that high levels of metallothionein would be present in tissues which were undergoing rapid growth and development, in order to supply zinc (and possibly copper) for nucleic acid metabolism, protein synthesis, and other metabolic processes. In this report we present the data for (zinc,copper)-thionein in neonatal and developing rat kidney from 2 to 70 days of age and rat testis from 10 to 70 days of age.

Terao and Owen (6) have studied copper metabolism in rat pups up to 42 days of age and have observed an increase in kidney total copper with age; in the present study, this increase is shown to be due to an increase with age in the amount of copper associated with metallothionein. Oh and Whanger (7) have reported the concentration of total zinc and zinc thionein in rat kidneys for five time points up to 90 days of age. Wong and Klaassen (8) have applied the 353Hg displacement technique of Piotrowski et al. (9), which provides no information about the complement of bound metallic ions, to the qualitative measurement of the metallothionein levels in rat kidney at eight time points up to 70 days of age.

Under normal conditions zinc thionein has been detected in the testis of the rat (10), while under abnormal conditions of cadmium administration, 109Cd-labeled metallothionein has been detected upon Sephadex G-75 column chromatography of rat and mouse testicular cytosol (11–13). The testes are the most rapidly growing tissue in young rats, as measured by the increase in number of cell nuclei (14), and, thus, might be expected to contain high levels of zinc thionein. This has been confirmed: 35- to 50-day-old male rats actually have more total zinc thionein in their testes than is present in their livers and kidneys combined. During the peak growth period (23 to 45 days) between 35 and 45% of testicular cytosolic zinc is associated with metallothionein, indicating the major involvement of this protein in zinc metabolism in this tissue. Little or no copper is associated with testicular metallothionein.

In this report total, particulate, cytosol, and metallothionein
zinc and copper concentrations have been determined in rat kidneys and testis during growth and development.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

Rat kidney metallothionein does not undergo the rapid and dramatic changes in concentration during neonatal and subsequent growth and development that occur in the liver (4), in the gastrointestinal tract (5), and in the testis (this study) of rats, and which might be correlated with changes in mitotic activity. The concentration fluctuates during these periods within limits. Our more complete data is consistent with earlier preliminary studies on rat kidney metallothionein (7, 8). Our studies also show that there is a slow and gradual accumulation in the growing rat kidney of copper which begins in the gastrointestinal tract (5), and in the testis (this study) at weaning. Much of this copper is in the cytosolic fraction, kidneys and testis during growth and development.

The concentration fluctuates during these periods. Confirming evidence that the testicular zinc binding protein detected in this study is indeed a metallothionein is provided by its behavior on molecular sieving and anion exchange chromatography and on disc polyacrylamide gel electrophoresis, and its labeling with [35S]cystine and nonlabeled with [3H]leucine in vivo. In terms of the organ content of metallothionein in adult rats (up to 70 days of age) the testes rank first, followed by the kidneys, the liver, and the gastrointestinal tract. As more tissues are examined during growth and development, the presence and importance of zinc thionein in these processes is likely to be substantiated further.

Evident also from the present studies is that the predominant isomethallothionein species in neonatal rat kidney and testis is zinc thionein II, as assessed by its behavior on anion exchange chromatography and on disc polyacrylamide gel electrophoresis. In older animals both isomethallothioneins I and II are present in comparable amounts in kidney, as observed previously by others (see Ref. 21 for example), but the testis continues to contain mostly zinc thionein II. This phenomenon does not occur in neonatal rat liver (4), but it does occur in regenerating rat liver (22) and in the livers of rats recovering from sham operation for adrenalectomy. Such observations raise interesting speculations on the presence and function of two isomethallothioneins, particularly as the displacement of zinc and other cations from metallothionein by mercury in vivo results in the generation of multiple species which migrate on anion exchange chromatography with decreased charge (23). Are isomethallothioneins I and II truly isoproteins, or are they the same protein with different metal compositions?

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K. Cain and B. Griffiths, manuscript in preparation.

**SUPPLEMENTAL MATERIAL**

**Metabolism of Zinc and Copper in the Neonate.** (Zn,Cu)-Thionein in the Developing Rat Kidney and Testis

Frank D. Brady and Michael Wamb

This section contains Experimental Procedures, Results, Figures, and a Table

**EXPERIMENTAL PROCEDURES**

Female Sprague-Dawley rats, maintained on a standard laboratory diet (PMI 1214), were reared overnight. After observation of a vaginal plug, the gravid females were housed in groups of six until day 23 of their pregnancy, at which time they were placed in individual plastic cages. First litters only were used in these studies. After birth mothers and pups were kept in the same cages without litter changes until weaning at 21 days of age. When neonatal pups (7 to 9) from a single litter were used for pooling of Zn and Cu determinations, the number of pups in each litter was counted and the mean of the number of pups in each litter was used. For early time points four pups were left with each mother, and the remainder of the litter was culled. For each data point a minimum of four litters was used. To prevent simplification, only the initial and final litters were used for each maternal group. The growth curves and tissue weights were determined on samples of maternal tissues. Complete metabolic parameters for each maternal group were calculated and the results were expressed as means ± SEM. Weights of mothers and pups were recorded and recorded as days. Male and female pups were weighed in the same cage without litter changes until weaning at 21 days of age. Male and female pups were then weighed separately. Ten and after weaning males were separated. For analysis of the paired t-tests, the mean 4 days of age was used.

**RESULTS**

Growth curves: Rat kidneys

<table>
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<th>Age (days)</th>
<th>Weight (g)</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
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<td>60</td>
<td>35.0</td>
</tr>
</tbody>
</table>

**FIG. 2.** Rat growth curves (left) and body weight (right) of rat kidney (left) and) body weight changes in the developing rat kidney (right) and testis (right) as a function of age after birth.

In Figures 1 to 5 are shown the age and age-related changes in the developing rat kidney and testis as a function of age after birth. Total, total, and metallothionein content was measured directly, with particulate zinc and copper being calculated as total metallothionein. Two significant differences were observed when the animals were studied with the use of two animals. The amount of metals in the neonate was measured as percent from litter to litter for a given age. This was probably due to a number of factors, including the amount of metals that are present in the maternal diet and the amount of metals that are transferred from the mother to the neonate. This was analyzed using two animals for each maternal group.

**FIG. 3.** Zinc and copper content in rat kidney (Zinc-Copper) and concentration in rat kidney (Zinc-Copper) as a function of age after birth. Total, total, and metallothionein zinc and copper were measured directly, with particulate zinc and copper being calculated as total metallothionein. Two significant differences were observed when the animals were studied with the use of two animals. The amount of metals in the neonate was measured as percent from litter to litter for a given age. This was probably due to a number of factors, including the amount of metals that are present in the maternal diet and the amount of metals that are transferred from the mother to the neonate. This was analyzed using two animals for each maternal group.
(Zn,Cu)-Thionein in Rat Kidney and Testis

**FIG. 5.** Zn (red) and copper (blue) concentration in rat testis: total, particulate, cytosolic, and metallothionein (Zn or Cu) bound, as a function of age after birth. Data expressed as metal per g tissue wet weight.

**TABLE 1** compares the total and metallothionein copper concentrations and contents in female and male kidneys. In terms of concentration, both total copper and metallothionein bound copper are significantly higher (P < 0.05) in females than in males at all ages. In terms of content, however, there are no significant differences between males and females at any age. This may be due to the different physiological states of the kidneys in the two sexes at different ages. Table 1 shows the mean values for each age group.

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Female</th>
<th>Male</th>
<th>Total Copper</th>
<th>Metallothionein Copper</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
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<td>1.54</td>
<td>2.02</td>
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</tr>
<tr>
<td>30</td>
<td>1.45</td>
<td>1.54</td>
<td>2.02</td>
<td>1.29</td>
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</tbody>
</table>

*Values are the means for 4 animals.*

The association of zinc and copper with metallothionein was studied in a number of ways, including the use of different methods of characterization. The results are shown in Figures 9 and 10, which illustrate the results for the characterization of zinc and copper in the kidneys of rats at different ages. Figure 9 shows the increased zinc and copper content in metallothionein from 20 to 30 days of age, as observed by thin-layer chromatography (TLCH). These results indicate that the metallothionein content increases with age and that the increase is more pronounced in the kidneys of male rats than in those of female rats. Figure 10 shows the increased zinc and copper content in metallothionein from 30 to 40 days of age, as observed by thin-layer chromatography (TLCH). These results indicate that the metallothionein content increases with age and that the increase is more pronounced in the kidneys of male rats than in those of female rats. The increase in metallothionein content is correlated with the increase in zinc and copper content, as observed by thin-layer chromatography (TLCH). These results indicate that the metallothionein content increases with age and that the increase is more pronounced in the kidneys of male rats than in those of female rats.
In 35 day old rat testes, zinc thionein II, unlike liver in which comparable amounts of metallothioneins I and II are known to exist (2,3). In Figure 10 is shown a DEAE-cellulose profile of metallothionein from the testes of rats, receiving [55]cysteine and [55]leucine. Metallothioneins are known to be rich in cysteine, but to contain little or no leucine (I). That the zinc protein in testicular cytosol is indeed a metallothionein is further supported by the incorporation of [55]cysteine into metallothionein I and II.

Several groups have reported the presence of a low molecular weight, [55]cysteine-binding protein in rat testes after in vivo treatment with [55]cysteine (11,12,28,30). This finding was probably due to binding of [55]cysteine to the endogenous zinc thionein present in mature rat testes, as shown by the experiments illustrated in Figure 11. In Figure 11A is shown a Sephadex G-25 profile of testicular soluble fraction after in vivo treatment with [55]cysteine, and in Figure 11B is shown a similar profile for the soluble fraction from control rats that was treated identically, except that cysteine was replaced by [55]cysteine. Most of the low molecular weight, [55]cysteine-binding protein fraction eluted from Sephadex G-25 in the same position as the zinc thionein.

The difference between the profiles of cysteine binding in Figure 11A and 11B is difficult to explain. It could possibly reflect differences in the zinc content of testicular cytosol in rats differing in age or in zinc content of the diet, or it could be due to differences in the metabolism of cysteine in testicular cytosol in rats differing in age or in zinc content of the diet. Metallothionein I is known to be rich in cysteine, but to contain little or no leucine (I). That the zinc protein in testicular cytosol is indeed a metallothionein is further supported by the incorporation of [55]cysteine into metallothionein I and II.

That the zinc protein in testicular cytosol is indeed a metallothionein is further supported by the incorporation of [55]cysteine into metallothionein I and II.

Zinc and Copper
(Ug tissue)
Fraction number

FIG. 95. Sephadex G-75 column chromatography of cytosol from 35 day old rat testis. Zinc (---), copper (--). Hb indicates the peak of elution of hemoglobin. Metallothionein elutes in fractions 27-33.

Zinc (ug/ml)

Fraction number

FIG. 9B. DEAE-cellulose column chromatography of the pooled zinc thionein from the G-75 columns. Zinc (---). Zinc II elutes in fractions 10-22 and zinc thionein II elutes in fractions 48-68.

Zinc (ug/ml)

Fraction number

FIG. 10. DEAE-cellulose column chromatography of cytosol from 35 day old rat testis from animals which had received [55]cysteine and [55]leucine four hours before killing. [55]cysteine, [55]leucine, (---).

Zinc (ug/ml)

Fraction number

FIG. 11. Sephadex G-15 column chromatography of cytosol from rat testes after (A, top) administration of [55]cysteine in vivo and (B, bottom) treatment with [55]cysteine in vitro. Hemoglobin (---) elutes in fraction 40. The peak of elution of soluble fraction, equivalent to 1 g tissue wet weight, from pooled testes (0.83 g tissue wet weight) was chromatographed. In (B) the soluble fraction from 0.15 g tissue wet weight was chromatographed. Procedures for details of treatment.