Induction of Metallothionein-Like Cadmium-Binding Protein in the Testis by Oral Cadmium Administration in Rats

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Abstract: The possible induction of a metallothionein (MT)-like Cadmium (Cd) binding protein (MT-like Cd-BP) was investigated in rat testis after oral Cd administration. Male Wistar rats were given Cd by oral administration (20 mg Cd/kg, for 10 weeks), while the experimental controls were given Cd by intraperitoneal (ip) injection (2 mgCd/kg). Cd concentration increased in the testes after both administrations. However, much more Cd (about 4 times) accumulated in the testes of rats receiving oral Cd administration than in rats receiving Cd ip injection (experimental control). Meanwhile, MT-like Cd-BP decreased dramatically in the testes after Cd ip injection compared to that in the testes of untreated control rats. However, this testicular MT-like Cd-BP after oral Cd administration increased significantly up to about 1.4 times of the amount found in the testes of untreated control rats. Inhibition of glutathione S-transferase (GST) activity and decreased glutathione (GSH) in the testes was not observed in rats after oral Cd administration. However, enzyme activity and GSH concentration were inhibited and decreased significantly in the testis by Cd toxicity after Cd ip injection. These results indicate that testicular MT-like Cd-BP, assumed to be MT and to be hardly inducible by Cd, is an inducible protein corresponding to increased Cd accumulation in the testis without damage by Cd toxicity after oral Cd administration.

Key words: Cadmium, Oral administration, Metallothionein (MT), Metallothionein-like cadmium binding protein (MT-like Cd-BP), Glutathione, Testicular cadmium toxicity

Introduction

The testis, like the lungs, is very sensitive to acute cadmium (Cd) toxicity. It is well known that Cd results in testicular damage such as hemorrhagic inflammation, atrophy, and necrosis or mortality1-5. Moreover, it is also known that toxic effects of Cd can be counteracted by administering selenium simultaneously with Cd6-7 or by pretreatment with a small dose of Cd before the challenge Cd administration4,5,8. Furthermore, it has been reported that the testicular damage mentioned above and the inhibition of enzyme activity in the testis are protected remarkably as a result of Cd binding by metallothionein (MT) in the testis6-7.

MT, a low molecular weight metal binding protein that has a high cysteine content (about 30 % in amino acid composition), is thought to play a role in the homeostatic control of zinc (Zn) and copper (Cu) metabolism, and detoxify the toxicity of certain heavy metals such as Cd9. It has been suggested that MT participates in the protection mechanism of the testis against Cd toxicity. The amounts
of MT-like Cd-BP as well as MT were quantified by the Cd-Hem method\(^{10}\) in various tissues including testes from rodents\(^{11,12}\). Onosaka and Cherian\(^{11}\) have reported the interesting results that MT in the testes decreased markedly in inverse proportion to the increase of Cd accumulation. Generally, MT is induced proportionally to the increased Cd accumulation in various organs including the liver and kidneys, but not in the case of the testis. The possibility of MT existing in the testis has been supported mainly, for example, immunohistochemical localization and the expression of MT mRNA\(^{13-10}\). Moreover, it has also been suggested that MT is locally synthesized in the testis, since it is well known that liver cells and other cells can induce MT\(^{17,18}\)

Although the several studies mentioned above have indicated the presence and the possibility of MT induction in the testis, they have indicated that the major Cd-BPs in the testis are not MT, but rather MT-like Cd-BP because this Cd-BP in the testis is not similar to MT in the liver or kidneys, as evidenced by the amino acid composition, u.v.-absorption spectra, and decreased induction by Cd injection\(^{15,19-25}\). Furthermore, it has recently been reported that purified Zn-BP in the testis has a different amino acid composition than MT, and that the antiserum acting against this protein is not cross-reacted with MT from the liver or kidneys in humans\(^{15}\).

Despite the numerous studies conducted on testicular Cd-BP or MT, there is still no consensus on whether testicular Cd-BP is inducible. Moreover, it is unclear whether this testicular Cd-BP is inducible. Furthermore, it is not known why the amounts of MT and MT mRNA increase in the testes under certain physiological conditions\(^{16,26-28}\). Whether or not this protein is MT, it is an important to clarify the possible induction, physiological role, and protective role of MT-like Cd-BP against Cd toxicity.

In the present study, we report the induction of MT-like Cd-BP, which is hardly inducible by Cd, in the testis by oral Cd administration.

We used the term of MT-like Cd-BP for testicular Cd-BP, since it has a different amino acid composition from MT\(^{15,19-25}\). Nevertheless, it should be noted that this Cd-BP is similar to MT in terms of its behavior on Sephadex G-75 gel filtration, heat stability, and metal binding\(^{9-18,22}\).

### Materials and Methods

1) Animals and treatments

Male Wistar rats were purchased from Clea Japan Inc. (Tokyo). The animals were fed commercial pellets (CE-2, Clea Japan Inc.), were given water ad libitum in temperature- and humidity-controlled rooms, and were kept on a 12 hr light/dark cycle during the experiment. From six weeks of age, animals were given Cd (CdCl\(_2\)) through a gastric tube at a dose of 20 mgCd/kg/day conservative six days a week for 10 weeks (the oral Cd administration group; n=5 each), and were injected intraperitoneal (ip) with 2 mgCd/kg (the Cd ip injection group as experimental control; n=5), and given distilled water (the control group; n=5 each). The animals in the oral Cd administration group were sacrificed after 0, 5, and 10 weeks of Cd administration. On the other hand, the animals in the Cd ip injection group were sacrificed 24 hr after Cd injection. All animals were sacrificed under light pentobarbital sodium anesthesia. Blood samples were drawn directly from the heart and the animals were killed by exsanguination. Then the blood remaining in the organs was washed out by perfusion via the injection of ice-cold saline solution. Testes were removed immediately, and homogenized in a 0.25 M Sucrose (pH 7.4) solution using a Polytron homogenizer. Homogenate was centrifuged at 105,000 g for 60 min at 4°C. A cytosol sample was used for determination of the enzyme activity and MT-like Cd-BP.

2) Determination of MT-like Cd-BP in the testis

The amount of MT-like Cd-BP was measured by the Cd-Hem method\(^{10}\). Homogenate (about 20%) of the testis in a 0.25 M sucrose solution was centrifuged at 13,000 rpm for 15 min. A supernatant of 200 µl was incubated with 1 ml of Cd solution (10 µg/ml) and 1 ml of 10 mM Tris-HCL (pH 8.6) buffer at room temperature. Then hemolyzate solution of 200 µl was added, mixed, boiled for 2.5 min, and centrifuged at 3,000 rpm for 5 min. This process was repeated three times. Finally, the supernatant was centrifuged again at 13,000 rpm for 5 min, and the Cd concentration was analyzed using an atomic absorption spectrophotometer to measure the amount of MT-like Cd-BP.

3) Gel-filtration chromatography

For gel filtration, a portion of the testicular cytosol (1.7 ml) was applied to a column (1.5 x 75 cm) of Sephadex G-75 previously equilibrated with 10 mM Tris-HCL, pH 8.6, containing 0.05 M NaCl and 0.02% NaN\(_3\), and fractions were eluted at a flow rate of 17.5 ml/hr at 4°C. Samples (1.7 ml each) were collected and determined OD at 250 nm and 280 nm, then analyzed for Cd, Zn, and Cu. MT fraction on gel-filtration was calibrated using MT obtained from the liver as a standard.
4) Biochemical estimation

Enzyme activities, Glucose 6 phosphate dehydrogenase (G6PDH; EC 1.1.1.49), Glutathione S-transferase (GST; EC 2.5.1.18), were determined respectively for the testicular cytosol sample to evaluate the effect of cadmium toxicity. The concentrations of reduced glutathione (GSH) and protein were also determined.

5) Metal determination

Concentrations of Cd, Zn, Cu, and iron (Fe) in the testis were determined using an atomic absorption spectrometer (Hitachi Zeeman 180-80), applying the flame and flameless methods after tissue digestion with nitric acid.

6) Statistical analysis

The data was analyzed statistically by the Student’s t-test and the ANOVA-Scheffe test at p<0.05.

Results

Hemorrhagic inflammation and increased testis weight (about 1.1 times: 2.25 ± 0.09 g to 2.06 ± 0.08 g in control rats) caused by Cd toxicity were observed in the rats after Cd ip injection. These findings were not observed in the testes after oral Cd administration (unpublished data). The Cd concentration in the testis after oral Cd administration was four times greater (1.7 µg/g to 0.4 µg/g) than that of rats receiving Cd injections (Fig. 1-A). On the other hand, the Cu concentration decreased significantly in the testes 24 hr after Cd ip injection and after 5 and 10 weeks of oral Cd administration (Table 1). The Fe concentration was significantly high in the testes resulting in hemorrhagic inflammation caused by Cd toxicity after Cd ip injection. On the other hand, Fe in the testes after oral Cd administration decreased significantly compared to Fe in the testes of untreated control rats. However, the Zn concentration did not change in any of the testes, including those of the untreated control rats (Table 1). The elution profile of Cd, Zn, and Cu in the testicular cytosol on Sephadex G-75 gel-filtration is shown in Fig. 2. Two peaks of Cd were eluted in the testicular cytosol of rats after Cd ip injection. A relatively large amount of Cd was localized mainly in the MT fraction, while a small amount was localized in the high molecular weight fraction. Moreover, a big Cd peak in the MT fraction was found in the testicular cytosol of rats after oral Cd administration. The distribution of Zn and Cu in the testicular cytosol was almost the same in the control and experimental groups. However, Cu in both fractions with a high-molecular weight and MT decreased in the testicular cytosol 24 hr after Cd ip injection (Fig. 2). MT-like Cd-BP in the testes of untreated control rats existed normally at high level (105.4 ± 7.4 µg/g-tissue). This testicular MT-like Cd-BP decreased significantly following Cd ip injection (64.0 ± 4.1 µg/g-tissue). However, the amount of MT-like Cd-BP in the 5th week after oral Cd administration maintained at the same level as in untreated control rats (123.9 ± 19.6 µg/g-tissue). Furthermore, this testicular MT-like Cd-BP increased significantly up to about 1.4 times amount of that in untreated control rats after 10 weeks of Cd administration (145.8 ± 8.0 µg/g-tissue). Namely, the testicular MT-like Cd-BP was induced corresponding to the increased Cd accumulation in the testis by oral Cd administration (Fig. 1-B).

![Fig. 1. Cadmium and Metallothionein-like cadmium-binding protein concentrations in the testis after the intraperitoneal injection and oral administration of cadmium](image-url)

Symbols: ■ Control; Untreated, 24 hr, 5, 10 week; Distilled water administration. 2 mgCd/kg ip injection. ■ 20 mgCd/kg oral administration. *: Significantly different to the control at p<0.05 (n=5).
To evaluate the toxic effect of Cd accumulated in the testis, GSH and the activity of enzymes (G6PDH, GST), related glycolysis, the pentose phosphate cycle, and detoxification with GSH in the testes were determined. The concentration of GSH decreased significantly in the testis after Cd ip injection, but not after oral Cd administration (Fig. 3-A). The activity of GST was also inhibited significantly in the testis after Cd ip injection (Fig. 3-B). While the activity of G6PDH in hemorrhagic testis increased markedly after 24 hr of Cd ip injection (Fig. 3-C). GST activity and GSH concentration in the testes after oral Cd administration were not inhibited and did not decrease, remaining at the same level as in untreated control rats (Fig. 3).

**Discussion**

To date, there have been many studies being done by the means of parenteral Cd administration to induce MT-like Cd-BP or MT in the testis, and to clarify the mechanism of toxic Cd effects or the protection mechanism against acute Cd toxicity. However, it seems to be difficult to control the Cd dose for the induction of MT-like Cd-BP or MT in the testis without any toxic effect by injecting Cd, is, 24, 32. The reason for this is that the testes are very sensitive to Cd toxicity. In the present study, we used oral Cd administration to maintain the necessary physiological conditions for Cd uptake from the gastrointestinal tract and Cd accumulation in the testis.
Severe hemorrhagic inflammation and edema by Cd toxicity were observed macroscopically in the testis after Cd ip injection, just as in our previous study, but not in the 5th and 10th weeks after oral Cd administration. The weight of hemorrhagic testes increased compared to that of the testes of the control rats. In contrast to the testicular damage resulting from Cd ip injection, Cd administered orally had no prominent effect on testicular weight or gross morphology, even though much more Cd was accumulated than in Cd injected rats (Fig. 1-A and unpublished morphological data). Namely, the Cd concentration in the testes after 10 weeks of oral Cd administration increased to about four times greater than in the testes of rats injected with Cd (Fig. 1-A).

Previous studies have reported that Cd accumulation in the testis after Cd injection was not high enough to cause damage resulting from severe Cd toxicity. Moreover, it has been thought that Cd induces testicular toxicity at a considerably lower dose than other organs such as the liver and kidney. For this reason, the testis is considered particularly susceptible to Cd toxicity, and more Cd can't be taken up into the testis. Furthermore, it has been reported that the testicular concentration of MT (the same as MT-like Cd-BP) in untreated normal testis is 5–20 times higher than in the liver and kidneys. However, the amount of this MT-like Cd-BP decreased significantly in the testis after Cd ip injection. Nevertheless, in the present study, much more Cd accumulated into the testis in the form of MT-like Cd-BP, which does not cause toxic damage. This indicates that there is no inhibition of enzyme activities and no decreased GSH in the testis (Fig. 1-B & 3, Table 1). These results suggest that the normal physiological function of the testis is maintained for the induction of MT-like Cd-BP by Cd. As a result, MT-like Cd-BP was induced significantly in the 10th week after Cd administration. However, in the 5th week, the amount of MT-like Cd-BP in the testes was not high, compared to that of untreated control rats. The amount of MT-like Cd-BP induced by oral Cd administration was not so great compared to the amount in the liver and kidneys. However, it was significantly increased (about 1.4 times than the level in untreated control rats). These results suggest that the ability of the testis to induce MT-like Cd-BP synthesis corresponding to the Cd accumulation may not be sufficient compared with that of the liver and kidneys, although the amount of pre-existent MT-like Cd-BP in the testis was extremely high. In other words, it is considered that Cd taken up into the testis was bound at first by pre-existing MT-like Cd-BP until saturation with Cd, then MT-like Cd-BP was induced moderately corresponding to the increase of Cd without testicular damage caused by Cd.

On the other hand, GSH is the most important nonprotein thiol, since it protects cells from damage by radiation, oxygen radicals, heart, and sulphhydril reactive agents, and provides...
the bulk of sulfhydryl groups for the detoxification of electrophilic xenobiotics\(^{32}\). In addition to MT or MT-like Cd-BP, it has been thought that GSH provides a buffer against Cd toxicity\(^{35}\). The elevations and the reductions in tissue GSH concentrations can reduce and exacerbate, respectively, the hepatotoxic and nephrotoxic effects of Cd\(^{36-38}\). Thus, GSH as well as MT play an important role in the cellular defense mechanism against toxic metals such as Cd. In the present study, decreased GSH concentration was observed in the damaged testes resulting in hemorrhagic inflammation and edema caused by Cd toxicity. Additionally, inhibited or increased enzyme activities were also found in the damaged testes. However, in rats after oral Cd administration, toxic damage such as hemorrhagic inflammation and decreased GSH was not observed in the testes where the level of induced MT-like Cd-BP was maintained. From the results, we would like to suggest that MT-like Cd-BP in the testes, being hardly inducible by Cd, is an inducible protein under certain physiological conditions without toxic damage by Cd.

Furthermore, the concentrations of Zn and Cu in the testis were monitored, as was MT synthesis, considering their relation to MT-like Cd-BP synthesis. Cu concentration, having a stronger affinity to MT than Cd, decreased according to the increased Cd accumulation in the testis after both Cd exposures (Fig. 2, Table 1). This result is interesting for what it reveals about the relationship between MT and MT-like Cd-BP regarding affinity to Cu. Prior to this, it had been thought that Cd binding to MT is affected by coexisting Cu having a stronger affinity than Cd\(^{6,13,15}\). However, based on the results of Sephadex G-75 gel filtration (Fig. 2), this Cu decrease in the testes seems to be the result of the indirect effect of Cd on Cu metabolism rather than of the replacement of Cd with Cu in the MT fraction.

The induction of MT-like Cd-BP and MT in the testis has been suggested as the one important factor in the protection mechanism\(^{8}\). However, it has been recently reported that MT (just like MT-like Cd-BP) did not participate in the protection mechanism against acute Cd toxicity in the testis, and that the changes in the testicular MT were dependent on Zn, not on the Cd concentration in the MT fraction\(^{39}\). However, the Zn concentration did not change significantly like Cu did in the present study. In other words, Zn metabolism seemed to be more stable than the response of Cu metabolism to Cd accumulation as MT-like Cd-BP in the testes. These results suggest that not only Cd and Zn, but also Cu, must be considered to clarify the significance of the existence and mechanism of MT-like Cd-BP synthesis in the testis.

In conclusion, Cd accumulation in the testis by oral Cd administration did not result in any toxic effect caused by Cd such as, for example, hemorrhagic inflammation, edema, inhibited enzyme activity, and decreased GSH. It was clarified that MT-like Cd-BP in the testis is an inducible protein under more normal physiological conditions by oral Cd administration. Additionally, the role that this testicular MT-like Cd-BP plays in preventing against Cd toxicity was also suggested.

However, further detailed studies, including the clarification of the amino acid sequence and gene expression of MT-like Cd-BP, are needed to clarify whether testicular MT-like Cd-BP is MT or Cd-BP, given the relation to the metabolism of Cu and Fe in the testes.

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**References**


