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INDUCTION OF ZINC-THIONEIN BY ESTRADIOL AND PROTECTIVE EFFECTS ON INORGANIC MERCURY-INDUCED RENAL TOXICITY

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Abstract—The castrated or unoperated male rats received an intravenous injection of $HgCl_2$ at a dose of 0.7 mg/kg of body weight (b.w.) after pretreatment with 30% ethanol or estradiol dissolved in 30% ethanol at a dose of 0.5 mg/kg b.w. subcutaneously twice a day for six consecutive days. Renal total protein, γ-GTP and K excretion in the rats treated with Hg and estradiol were significantly lower than the corresponding values in the rats treated with Hg alone, suggesting that pretreatment with estradiol ameliorates the renal toxicity of Hg in male rats. Pretreatment with estradiol significantly increased Hg and Hg-thionein(Hg-MT) concentrations in the renal cortex of the animals treated with Hg, though in the liver this agent did increase the Hg-MT without elevation of Hg concentration. Treatment with estradiol alone (0.5 mg/kg, s.c., twice a day, for six consecutive days) significantly increased the zinc-thionein (Zn-MT) concentration in the kidney and liver. Simultaneous treatment with 10^{-5} M estradiol and Hg in human amniotic-fluid cells caused a significant increase in the uptake of Hg and the synthesis of Hg-MT, suggesting that estradiol may directly stimulate an accumulation of Hg into the cells and the synthesis of Hg-MT. Together, all of the above findings suggest that pretreatment with estradiol may increase the uptake of Hg, which in turn leads to the increase in the Hg-MT concentration. The induction of Zn-MT by pretreatment with estradiol may account for the protective effect of estradiol on Hg-induced renal toxicity.

The response of rats to minimum toxic doses of inorganic mercury (Hg) varies with the sex of the animals [1–4]. Treatment with female sex hormones protects the proximal tubular cells from necrosis usually noted when this dose of Hg is given alone in male rats [5], though testosterone markedly increases the renal toxicity of Hg [3–4]. However, the mechanisms by which female sex hormones protect the renal toxicity of Hg are not understood.

Studies of the toxicological function of the metal rich protein, metallothionein (MT), have focused on its role in the detoxication of cadmium (Cd) and mercury [6]. Metals and glucocorticoids, especially dexamethasone and hydrocortisone are known to operate as primary inducers of the synthesis of MT [7–10]. Karin and Herschman [11] have demonstrated that the induction of MT in HeLa cells by the steroid hormones is specific to the glucocorticoid class. In the only study of which we are aware, female sex hormones, especially estradiol are unable to induce the synthesis of MT [11]. However, MT concentration in the liver of female rats treated with Cd or Hg was higher than that in male rats [12–14]. Recently we have reported that pretreatment with estradiol increases the Cd-thionein (Cd-MT) concentration in the kidney and liver of the male rats treated with Cd though testosterone could not increase the Cd-MT [15]. These findings suggest that female sex hormones can induce the synthesis of MT in the liver and kidney, which in turn results in the protection against Hg-induced renal toxicity.

The data presented in this report demonstrate that a female sex hormone, estradiol, can influence the biosynthesis of both zinc-thionein (Zn-MT) and Hg-thionein (Hg-MT). The increased concentration of Zn-MT induced by pretreatment with estradiol may be a factor for the preventive action of estradiol on Hg-induced renal toxicity.

MATERIALS AND METHODS

Animals. Male STD–Wistar rats, weighing 220 g on an average, were purchased from Shizuoka Laboratory Animal Center and maintained on a commercial diet. They received water *ad libitum*. They were housed in a temperature- and light-controlled room as previously reported [16].

Hg *and estradiol injection.* Thirty-two male rats were divided into six groups: control rats (group 1), Hg-treated rats (group 2), estradiol-treated rats (group 3), Hg- and estradiol-treated rats (group 4), castrated rats treated with Hg alone (group 5) and castrated rats treated with Hg and estradiol (group 6). The rats of groups 2 (N = 6), 4 (N = 5), 5 (N = 5) and 6 (N = 5) respectively received an intravenous injection of $HgCl_2$ at a dose of 0.7 mg/kg of body weight (b.w.) after pretreatment with 30% ethanol (groups 2 and 5) or estradiol (groups 4 and 6) at a dose of 0.5 mg/kg b.w. subcutaneously twice a day for 6 consecutive days. The rats of groups 2 and 5

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§ Abbreviations used: estradiol, 1,3,5(10)-estratriene-3,17β-diol.

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were predosed with 30% ethanol because estradiol was dissolved in 30% ethanol. The rats of group 1 (N = 6) and 3 (N = 5) respectively received an injection of 0.1 ml of physiological saline after pretreatment with 30% ethanol (group 1) or estradiol (group 3) at a dose of 0.5 mg/kg b.w. subcutaneously twice a day for six consecutive days. The rats of groups 5 and 6 were castrated on day 3 prior to the injection of 30% ethanol (group 5) or estradiol (group 6). After 24 hr of treatment with Hg the rats placed in a metabolic cage were sacrificed. Injected solution of Hg (0.1 ml) contained labeled $^{203}HgCl_2$ at the final concentration of 15.0 μCi/ml (specific activity 8.3 mCi/mg; New England Nuclear).

Evaluation of Hg-*induced nephrotoxicity.* The concentration of urinary protein and the activity of γ-glutamyltranspeptidase (γ-GTP) were determined by using a commercial kit. The concentration of sodium (Na) and potassium (K) in the urine was determined by flame photometry.

Subcellular fraction. The renal cortex was homogenized in a Teflon–glass homogenizer, in 2.5 ml of ice-cold 0.25 M sucrose containing 0.02 mM Tris–HCl buffer (pH 7.6). The nuclear fraction was obtained at 700 *g* for 10 min. A large-granular fraction was obtained at 10,000 *g* for 10 min and washed. The washing was added to the supernatant. The remaining supernatant was centrifuged at 10,500 *g* for 60 min providing microsomal and cell supernatant fractions.

Chromatographic procedure. The renal cortex was homogenized in ice-cold 20 mM Tris–HCl buffer (pH 8.0) with proper bubbling with N_2 gas. The supernatant fraction was quickly applied to a column (2.6 × 100 cm) packed with Sephadex G-75 gel. The sample was eluted from the column with 20 mM Tris–HCl (pH 8.0) at a rate of 36 ml/hr. A fraction (3 ml) was collected and analyzed by γ-counting.

Assay of metallothionein (MT). Subcutaneous injection of 0.1 ml of estradiol in the backs of the animals at the three doses of 0.1, 0.5 and 1.0 mg/kg b.w. was performed twice a day (12-hr intervals) for six consecutive days. Control and ethanol-treated rats were given subcutaneously physiological saline (0.1 ml) and 30% ethanol respectively twice a day for six consecutive days. The animals were not starved overnight before being sacrificed. They were quickly decapitated between 0900 and 1100 hr. The livers and kidneys from the control and treated rats were removed and frozen quickly in liquid N_2. MT concentration in the kidney and liver was determined by the method of Cd saturation-hemolysate method as previously reported [17].

Culture of human amniotic-fluid cells. Upon reaching confluency, the cells were incubated in a culture dish (60 mm) containing Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal calf serum at 37°. The cells were washed with fresh serum-free medium before the exposure of Hg. The cells were incubated for 24 hr in 1.0 ml of serum free-medium containing 12 μCi per ml of $^{203}HgCl_2$ (specific activity 8.3 mCi/mg) and 10^{-5} M estradiol at the final concentration. Cells were harvested and the soluble proteins were chromatographed on Sephadex G75 (1.5 × 45 cm) as described above.

Statistical analysis. The data were evaluated by Student's *t*-test or one-way analysis of variance.

RESULTS

Renal total protein, γ-GTP and K excretion in the rats treated with Hg and estradiol were significantly lower than those in rats treated with Hg alone (Table 1). The pattern of renal total protein, γ-GTP and K excretion, but not of Na excretion, in the castrated rats treated with Hg and estradiol was similar to those of unoperated rats treated with Hg and estradiol. Renal total protein, γ-GTP and K excretion in the rats treated with estradiol alone were also significantly lower than the corresponding values in control rats.

Hg concentration in the whole kidney, renal cortex and renal medulla of the animals treated with Hg and estradiol was significantly higher than the corresponding values in the rats treated with Hg alone (Table 2). On the contrary urinary total Hg excretion in these rats was significantly lower than that of the rats treated with Hg alone (Table 2). The pattern of Hg concentration in the whole kidney, renal cortex and renal medulla of the castrated animals treated with Hg and estradiol was similar to those of unoperated rats treated with Hg and estradiol. Hg con-

Table 1. Effect of estradiol on urinary total protein, γ-GTP and K excretion in the male rats treated with Hg

	Protein (μg)	γ-GTP (mU)	Na (μEq)	K (μEq)
Control	8084 ± 898	226 ± 51	388 ± 70	1378 ± 154
Estradiol	932 ± 345*	56 ± 19*	301 ± 97	818 ± 315*
Hg alone	20726 ± 3591*	1313 ± 408*	576 ± 180	1749 ± 160
Hg + estradiol	3025 ± 731†	337 ± 119†	535 ± 140	1130 ± 259†
Castration and Hg alone	18555 ± 2640*	5465 ± 4001	1013 ± 128*	1762 ± 110
Castration and Hg + estradiol	3351 ± 1359†	708 ± 373†	396 ± 67†	702 ± 106†

The data are expressed as total content of protein (μg), γ-GTP (mU), Na (μEq) and K (μEq) in the urine per 24 hr period. The data are shown as $\bar{X} \pm SE$ of five animals. Significantly different from the control values at $P < 0.01$ (*). Significantly different from the values in the unoperated rats treated with Hg alone at $P < 0.01$ (†). The unoperated or castrated rats received an intravenous injection of $HgCl_2$ at a dose of 0.7 mg/kg of body weight (b.w.) after pretreatment with 30% ethanol or estradiol at a dose of 0.5 mg/kg b.w. subcutaneously twice a day for six consecutive days. The control or estradiol-treated rats received an injection of 0.1 ml of physiological saline after pretreatment with 30% ethanol or estradiol at a dose of 0.5 mg/kg b.w. subcutaneously twice a day for six consecutive days. Castration was carried out on day 3 prior to the injection of 30% ethanol or estradiol.

Table 2. Effect of estradiol on Hg concentration in the kidney, urine and liver of the rats treated with Hg

	Whole kidney	Renal cortex	Renal medulla	Liver	Urine
Hg alone	27.7 ± 0.9	33.5 ± 0.8	24.4 ± 1.3	1.1 ± 0.08	7.0 ± 0.9
Hg + estradiol	35.4 ± 4.5*	52.1 ± 3.5*	31.4 ± 4.5*	1.1 ± 0.2	2.2 ± 0.5*
Castration and Hg alone	30.6 ± 1.1	38.8 ± 2.4	30.7 ± 1.4*	1.3 ± 0.1	8.0 ± 1.3
Castration and Hg + estradiol	44.1 ± 1.4*	47.5 ± 2.9*	3.4 ± 1.7*	0.9 ± 0.1	2.4 ± 0.3*

The data are expressed as μg Hg per g of wet tissue and $\bar{X}$ ± SE of five animals. The values of urine are also expressed as the total content of Hg (μg) in the urine per 24 hr period. Significantly different from the values in the rats treated with Hg alone at $P < 0.01$ (*). Injection schedule is described in Table 1.

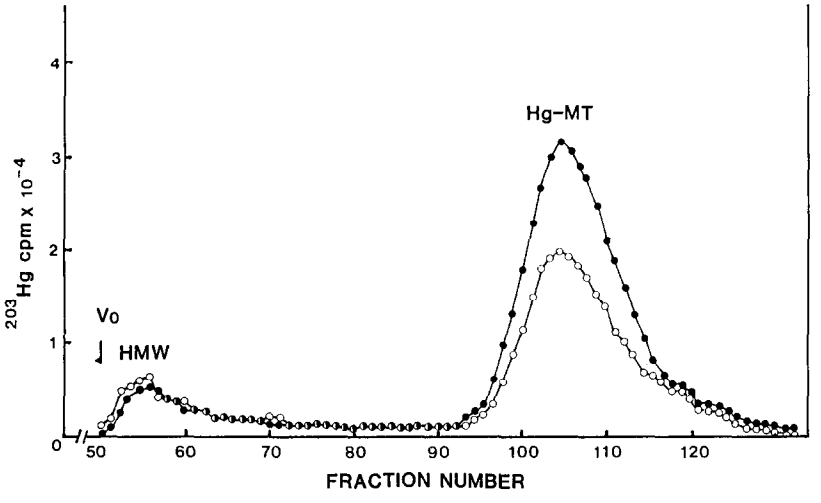

Fig. 1. Profile of Sephadex G-75 chromatogram of renal cortex of unoperated rats treated with Hg and estradiol (●) or treated with Hg alone (○). Injection schedule is described in Table 1. HMW, high molecular weight protein; Hg-MT, mercury-thionein; Vo, void volume.

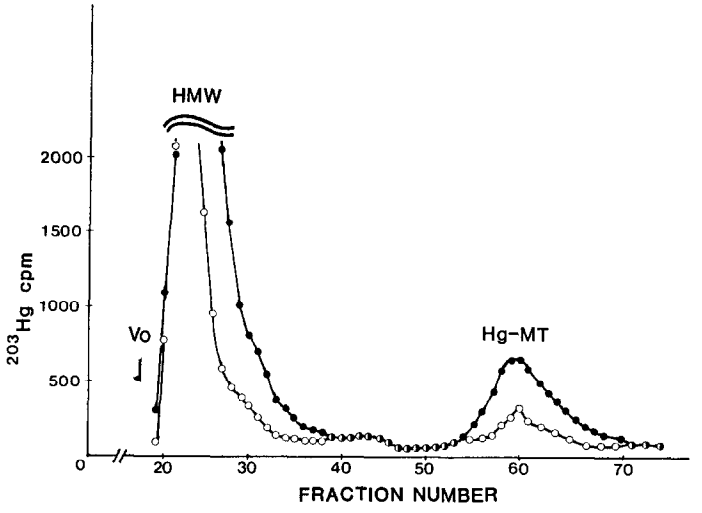

Fig. 2. Profile of Sephadex G-75 chromatogram of human amniotic fluid cells treated with Hg and estradiol (●) or treated with Hg alone (○). A quantity of 12 μCi of $^{203}HgCl_2$ was added to the serum free fresh culture medium in the presence of 10^{-5} M estradiol and the cells were incubated for 24 hr. Cells were harvested and the soluble proteins were chromatographed on Sephadex G-75 (1.5 × 45 cm). HMW, high molecular weight protein; Hg-MT, mercury-thionein; Vo, void volume.

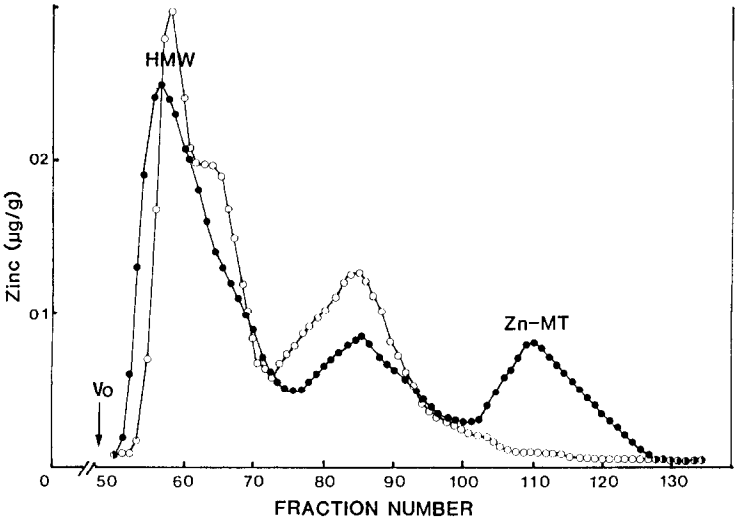

Fig. 3. Profile of Sephadex G-75 chromatogram of liver of unoperated rats treated with estradiol alone (0.5 mg/kg, s.c. twice a day for six consecutive days). ○, control rats; ●, estradiol-treated rats; HMW, high molecular weight protein; Zn-MT, zinc-thionein; Vo, void volume.

centration of the supernatant fraction, which contained the highest concentration of Hg among the subcellular fractions, from the renal cortex of the unoperated or castrated rats treated with Hg and estradiol was significantly higher than that in those rats treated with Hg alone (the data are not shown).

The gel filtration profile of the supernatant of the renal cortex from the rats treated with Hg alone shows two peaks of radioactivity (Fig. 1). The first and small peak, eluted in the void volume, consisted of high molecular weight proteins (HMW). The second radioactive peak, with an elution volume to void volume ratio of 2.0–2.2, corresponding to the major peak of protein-bound Hg is presumably associated with Hg-thionein (Hg-MT). Hg-MT concentration in the kidney of rats treated with Hg and estradiol was significantly higher than the corresponding value in the rats treated with Hg alone (Fig. 1). Incubation of human amniotic-fluid cells for 24 hr with 10^{-5} M estradiol and Hg caused a significant increase in the synthesis of Hg-MT (Fig. 2) and the accumulation of ^{203}Hg into the cells (the data are not shown). Gel filtration chromatography of the liver supernatant from the rats treated with estradiol alone (Fig. 3) revealed that in response to estradiol, there was an increase in the concentration of Zn-MT. Treatment with estradiol alone significantly increased the Zn-MT concentration in the kidney and liver (Table 3).

DISCUSSION

Sex related differences observed in mercury (Hg)-induced renal toxicity are well known [1–4]. Some workers have suggested that the high sensitivity of male rats to Hg is due to a male sex hormone, testosterone, which may be a determinant of sex differences in the renal toxicity of Hg [3–4]. Muraoka and Itoh [3] have suggested that the lower sensitivity of female kidney to Hg may not be determined by the female sex hormones since pretreatment with estradiol (0.2 mg/kg, daily for 7 consecutive days) does not protect the Hg-induced proximal tubular renal necrosis in the castrated rats. In our present study, renal total protein, γ-GTP and K excretion in the rats treated with Hg and estradiol were significantly lower than those in rats treated with Hg alone, suggesting that pretreatment with estradiol

Table 3. Effect of estradiol on the zinc-thionein concentration in the liver and kidney

	Kidney	Liver
Control	92.8 ± 4.3	20.8 ± 3.2
Ethanol	97.0 ± 1.9	40.0 ± 4.0†
Estradiol (0.1 mg/kg)	166.0 ± 5.9*	196.0 ± 18.8†
Estradiol (0.5 mg/kg)	181.0 ± 6.0*	216.0 ± 38.0†
Estradiol (1.0 mg/kg)	155.0 ± 2.1*	132.0 ± 15.0†

Zinc-thionein concentration is expressed as μg/g and $\bar{X}$ ± SE of five rats treated with a physiological saline (control) or 30% ethanol (ethanol) or estradiol (0.1 mg/kg, 0.5 mg/kg and 1.0 mg/kg respectively, s.c. twice a day for six consecutive days). Significantly different from the control value in the kidney at $P < 0.01$ (*). Significantly different from the control value in the liver at $P < 0.01$ (†). Injection schedule is described in Materials and Methods.

ameliorates the renal toxicity of Hg in male rats. Our present findings support Harber's histopathological findings indicating that pretreatment with estrogen prevents the proximal tubular renal necrosis associated with Hg [5].

It is well known that the induction of the synthesis of MT in the liver and kidney of adult animals provides a detoxification mechanism against acute exposure to cadmium and presumably, to other metals [6]. Hg concentration in the whole kidney, renal cortex and renal medulla of the animals treated with Hg and estradiol was significantly higher than the corresponding values in the rats treated with Hg alone. Hg-MT concentration in the kidney of rats treated with Hg and estradiol was significantly higher than the corresponding value in the rats treated with Hg alone. These results suggest that the increase in the synthesis of Hg-MT in the kidney of rats treated with Hg and estradiol is responsible for the increased concentration of Hg. We assumed that the increase in the synthesis of Hg-MT *in vivo* may be directly stimulated by treatment with estradiol. Incubation of human amniotic-fluid cells for 24 hr with 10^{-5} M estradiol and Hg caused a significant increase in the accumulation of ^{203}Hg into the cells and the synthesis of Hg-MT, suggesting that estradiol may directly stimulate Hg-MT synthesis. However, it remains to be determined that Hg-MT is induced as a result of an increase in the uptake of Hg.

Hg can rapidly and effectively displace Zn from MT [18]. We postulated that zinc-thionein (Zn-MT) induced by pretreatment with estradiol could be displaced by Hg, which in turn would lead to the increase in the concentration of Hg-MT. Gel filtration chromatography of the liver supernatant from the rats treated with estradiol revealed that in response to estradiol, there was an increase in the concentration of Zn-MT. Treatment with estradiol alone significantly increased the Zn-MT concentration in the liver and kidney. There are few data regarding the effect of glucocorticoids on the induction of Zn-MT in the kidney, though in the liver these agents are known to be the primary inducers of the synthesis of Zn-MT [6]. The present study demonstrates that a female sex hormone, estradiol, can induce the synthesis of Zn-MT in the kidney of male rats. Pretreatment with estradiol did increase the Hg-MT concentration without elevation of Hg concentration in the liver, though in the kidney this agent caused an increase in the concentration of Hg-MT with elevation of Hg concentration. Simultaneous treatment with estradiol and Hg also increased the uptake of Hg and the synthesis of Hg-MT in cultured human amniotic fluid cells. We have already shown that pretreatment with estradiol increases the Cd-MT and Cd concentration in the kidney, liver and heart of the male rats treated with Cd [15]. Together, all of the above findings suggest that pretreatment with estradiol directly increases the uptake of Hg, which in turn leads to the elevation of Hg-MT concentration.

It has been shown that the toxicity of a metal bound to MT becomes less toxic [6, 10]. A positive correlation between dose-related increases in hepatic Cd-MT and cadium LD_{50} suggests a protective factor for MT [19]. Koyama *et al.* [20] have suggested that the protective effect of the pretreatment with some metals such as calcium and nickel on Hg-induced toxicity in mice is associated with the increase in the concentration of renal MT.

The data presented in this report demonstrate that a female sex hormone, estradiol, can influence the biosynthesis of both zinc-metallothionein (Zn-MT) and Hg-metallothionein (Hg-MT). The increased concentration of Zn-MT induced by pretreatment with estradiol may be a factor for the preventive action of estradiol on Hg-induced renal toxicity.

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