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EFFECT OF SEX HORMONES ON THE FATE OF METHYLMERCURY AND ON GLUTATHIONE METABOLISM IN MICE

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Abstract—To investigate the mechanisms for the sex-related difference in the *in vivo* fate of methylmercury (MeHg), the effects of hormonal manipulation on the distribution and urinary excretion of the mercurial moiety (Hg) of injected MeHg and on hepato-renal metabolism of glutathione were studied in C57BL/6N mice. Twenty-four hours after oral administration of MeHg, urinary Hg levels were significantly higher in males than in females. Tissue Hg levels of males were higher in the kidney, but lower in the brain, liver and plasma than those of females. The fate of injected MeHg in castrated males was similar to that in normal females except for its brain levels. This feminization of the mercurial behavior in the castrated males was restored by treating with testosterone propionate (TP). When control mice were treated with TP, urinary excretion of Hg increased in both sexes, whereas renal Hg level increased only in females. Administration of estradiol benzoate (EB) to males decreased the renal accumulation and urinary excretion of Hg, whereas its hepatic levels increased. However, no significant change in the fate of MeHg was found in females pretreated with EB. Castration of females slightly decreased the urinary excretion of Hg. Thus, tissue distribution and urinary excretion of the administered MeHg seem to be subject to sex hormone control. Since MeHg has a high affinity for GSH, effects of hormonal manipulation on the metabolism of hepato-renal glutathione were also investigated. A significant sex-related difference in glutathione levels was found in plasma but not in the kidney, liver and erythrocytes. The half-lives of glutathione in the liver and kidney were significantly shorter in males than in females as determined by treatment with buthionine sulfoximine, a specific inhibitor of GSH synthesis. This difference was also modulated by the hormonal treatment. Since half-lives of GSH in the liver and kidney predominantly reflect the rate of its efflux from these tissues, the results suggest that GSH metabolism and/or secretory transport may be regulated by sex hormones. These and other observations suggest that the fate of MeHg may be modulated by way of regulating the inter-organ metabolism and transport of glutathione and its derivatives.

We recently demonstrated that mice show a marked sex difference in the distribution and urinary excretion of administered methylmercury (MeHg) and that this difference becomes obvious concomitant with their sexual maturation [1]. Renal accumulation and urinary excretion of Hg administered as MeHg occur much faster in males than in females. These observations drove us to speculate that a sex hormone-related mechanism may underlie the *in vivo* handling of MeHg. It is known that testosterone induces a dramatic alteration in the morphological and biochemical aspects of the renal proximal tubules [2–4]. Thus, renal function relating to MeHg excretion may also be affected by sex hormones.

It has been suggested that the formation of a MeHg–GSH complex is of importance for MeHg accumulation in the kidney [5]. Previous studies [6] revealed that administration of MeHg conjugate of GSH causes significantly higher accumulation of Hg in the kidney than when methylmercuric chloride alone or MeHg cysteine conjugate is administered. The liver has the ability to secrete many xenobiotics as their glutathione conjugates [7] via a carrier-mediated mechanisn [8, 9]. Since MeHg has a high affinity for thiol compounds such as GSH [10], the metabolism and transport of glutathione and its derivatives could be one of the most important factors for determining tissue distribution and elimination of MeHg in organisms challenged with this toxic compound. To investigate the mechanism for the sex-related difference, the effects of hormonal manipulation on tissue distribution and urinary excretion of MeHg and on glutathione metabolism in the liver and kidney were studied in male and female mice.

MATERIALS AND METHODS

Materials. Methylmercuric chloride (MMC) was purchased from the Wako Chemical Co. (Osaka); the purity of MMC used for the present experiments was higher than 98% as determined by electron capture detector (ECD)-gas chromatography. The mercurial was dissolved in highly purified water to

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give a concentration of 0.5 mg/ml. Testosterone propionate (TP, 1 mg/ml) and estradiol benzoate (EB, 0.1 mg/ml) in sesame oil injection solution were purchased from the Mochida Pharmaceutical Co. (Tokyo). DL-Buthionine sulfoximine (BSO) was synthesized and crystallized according to the method of Griffith and Meister [11]. The synthetic product was characterized by thin-layer chromatography on silica gel 60 in a solvent system of *n*-butanol/acetic acid/pyridine/water (15:3:10:12) which gave a single ninhydrin positive spot ($R_f = 0.38$). The purity of the product was higher than 99% (213–214°; decomposed, lit., 214–215°).

Animals. C57BL/6NJcl male and female mice (7 weeks of age) were used in the present experiments. Unless otherwise stated, five to seven mice were housed on corncob bedding in a clear plastic cage in an air-conditioned room maintained at 23 ± 2° with a light–dark cycle of 12/12 hr. Animals were allowed free access to laboratory chow (CE-2, Clea Japan) and water.

Hormonal treatment. Animals were castrated under pentobarbital anesthesia and allowed to recover for 10 days prior to experiments. TP or EB solution was injected subcutaneously daily into mice at a dose of 50 mg/kg or 1 mg/kg for 7 days respectively. Control mice were injected with the same volume of the vehicle seasame oil alone.

Analysis of mercury distribution and excretion. Control and hormonally treated animals were administered MMC orally at a dose of 5 mg/kg. Then, urine and feces were collected for 24 hr in metabolism cages (one mouse/cage). After 24 hr, blood was collected from the left femoral artery into heparinized test tubes under pentobarbital anesthesia. Then tissues were perfused with saline through the heart and excised. Aliquots of blood samples were centrifuged at 7000 *g* for 10 min at room temperature to separate plasma and blood cells. Total Hg contents of these samples were determined according to the oxygen combustion–gold amalgamation method [12] using a Sugiyamagen Mercury Analyzer (Tokyo, Japan). MeHg contents in the liver, kidney and urine samples were determined by ECD-gas chromatography.

GSH analysis. Hormonally treated and control animals were intravenously and intraperitoneally injected with BSO, a specific inhibitor of γ-glutamylcysteine synthetase [11], at a dose of 1 mmol/kg respectively. At appropriate intervals after administration (5, 15, 30, 45, 60 and 90 min) of BSO, blood samples were collected from the femoral artery and animals were killed. Total glutathione (TGSH) levels in the excised tissues were determined by the method of Teitze [13]. Half-lives of glutathione were estimated from the semilogarithmic plots of tissue TGSH levels before and after BSO treatment.

Statistical analysis. Statistical significance of difference was calculated according to Student's *t*-test; the level of significance was put at $P < 0.05$.

RESULTS

Effects of hormonal manipulation on MeHg metabolism. Figure 1 demonstrates the effects of hormonal modulation on Hg levels in urine and feces collected for 24 hr following administration of MMC in C57BL male and female mice. In the intact mice, males excreted 6.5 times higher Hg in urine than females. Treatment of animals with TP accelerated urinary excretion of Hg in males and females by a factor of 1.7 and 3.3 respectively. In contrast, treatment of animals with EB caused depletion of urinary Hg excretion in males by one-third. Castration of males decreased the urinary excretion of Hg to a level approaching that of the intact females. Treatment with TP in castrated mice increased the urinary excretion of Hg. In contrast to the marked difference in the urinary excretion of Hg in the intact mice, no significant difference was found in the fecal Hg levels between the two sexes. Hormonal manipulation failed to affect the fecal levels of Hg in both sexes.

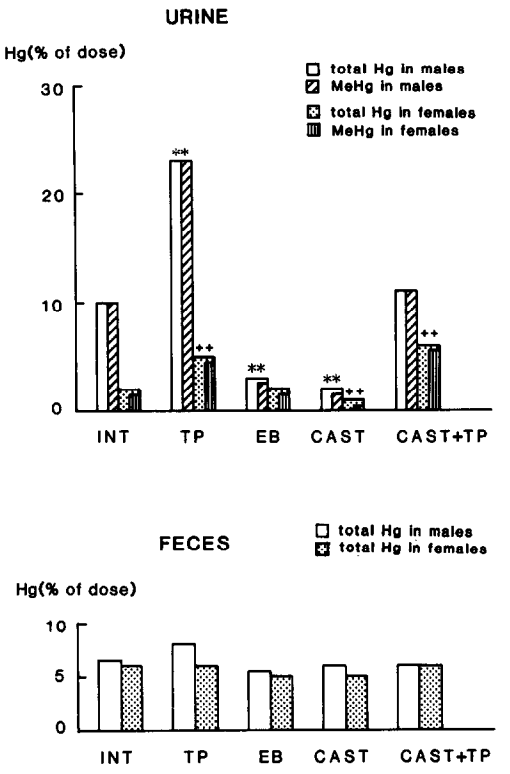

Fig. 1. Effect of hormonal manipulation on Hg excretion for 24 hr following methylmercuric chloride (MMC) administration. Administration of sesame oil (INT), testosterone propionate (TP), estradiol benzoate (EB) and castration (CAST) were carried out as described in the text. After each treatment, the mice were administered MMC (5 mg/kg body wt) orally. Urine and feces were collected for 24 hr, and their Hg levels were determined as described in the text. Mean values were derived from five experiments. There were significant differences ($P < 0.01$) in urinary Hg levels between the intact males and females. Key: ** significantly different ($P < 0.01$) from intact males, and ++ significantly different ($P < 0.01$) from intact females.

Figure 2 shows the effects of hormonal modulation on tissue Hg levels. The renal Hg levels were significantly higher in control males than in females, whereas Hg levels of the liver and brain were lower in the males than in the females. Treatment with TP

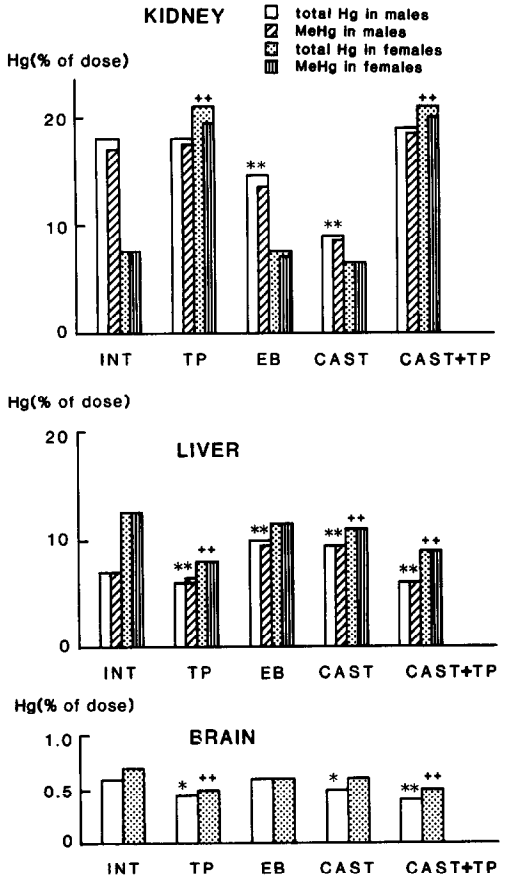

Fig. 2. Effect of hormonal manipulation on Hg distribution at 24 hr after MMC administration. Mice were treated as described in Fig. 1. Mean values were derived from five experiments. There were significant differences ($P < 0.01$) in Hg levels of the kidney, liver and brain between the intact males and females. Key: * significantly different ($P < 0.05$) from intact males, ** significantly different ($P < 0.01$) from intact males, and ++ significantly different ($P < 0.01$) from intact females.

increased renal Hg levels in females, but decreased the liver and brain Hg levels in both sexes. Hg distribution in the liver, kidney and brain of the castrated males was similar to those in the intact females. This feminization was reversed by treating the castrated males with TP. Treatment of animals with EB also decreased the renal Hg levels, and increased its hepatic levels in males but not in females. Most of the Hg in the liver and kidney was accounted for by an organic mercurial; no significant amount of inorganic mercurials was found in these tissues. Since Hg levels in the brain were significantly lower than those in other tissues, MeHg levels in this organ were not determined.

The effects of hormonal manipulation on Hg concentration in plasma and blood cells (mainly RBC) and on their ratio are shown in Table 1. Plasma concentration of Hg was lower in males than in females. However, no significant difference in the Hg concentration in RBC was found between the two animal groups. Thus, the plasma/RBC ratio for Hg distribution was lower in males than in females. Treatment with TP decreased the Hg levels of both plasma and RBC in females and of RBC in males. Both castration and treatment with EB of males elevated both plasma Hg levels and plasma/RBC ratio. Treatment of castrated males with TP restored plasma Hg levels toward that of the intact males and decreased Hg levels in RBC.

Effects of hormonal manipulation on GSH metabolism. Tissue GSH levels are determined by the dynamic equilibrium between its biosynthesis and utilization. Recent studies [14, 15] revealed that the efflux of GSH and its derivatives is the major determinant for GSH turnover in the liver and kidney. Thus, elimination of xenobiotics having high affinity for thiol compounds may depend on the turnover rate of tissue GSH. To test whether the sex difference in the fate of MeHg correlates with the difference in the mode of GSH metabolism, tissue levels and turnover rates of glutathione were compared between males and females. As shown in Table 2, a significant sex-related difference in TGSH levels was found in plasma but not in the kidney, liver and erythrocytes. On the other hand, experiments with BSO revealed that turnover rates of tissue TGSH in the liver and kidney were significantly different between the two animal groups; values of 57 and 18 min for males and 100 and 35 min for females were obtained for half-lives of TGSH in the liver and kidney respectively (Fig. 3). None of the hormonal manipulations caused significant changes in hepato-renal glutathione levels. The half-lives of TGSH in the hormonally manipulated groups were estimated from tissue TGSH levels before and after buthionine sulfoximine treatment, as shown in Fig. 3. Both castration and EB treatment of males increased the half-lives of hepato-renal TGSH to levels approaching those of females (Table 3). On the contrary, treatment of females with TP decreased the half-lives of hepato-renal TGSH. Similar treatment of males with TP did not alter the turnover rates of hepato-renal TGSH. Neither castration nor EB treatment of females induced a detectable change in GSH metabolism.

DISCUSSION

Previous studies [1] revealed that mice show a marked difference in the fate of administered MeHg between males and females. The present work demonstrates that the metabolism and elimination of MeHg occur significantly faster in males than in females and that the sequence of events leading to urinary excretion of MeHg may proceed under the control of sex hormones. Renal accumulation and urinary excretion of Hg in TP-treated females increased with concomitant decrease in Hg levels of the liver, brain and erythrocytes. These observations suggest that the transfer of MeHg from extrarenal tissues and cells to the kidney is important for its urinary excretion. In the case of TP-treated males, however, urinary excretion of Hg occurred with no significant change in its renal levels. Excess TP may enhance the urinary excretion of MeHg from the kidney.

It has been reported that MeHg has a high affinity

Table 1. Effect of hormonal manipulation on Hg levels in plasma and RBC, and plasma/RBC ratio of Hg*

		Hg		
	Treatment†	Plasma (μg/ml)	RBC (μg/ml)	Plasma/RBC
Males	Intact	0.58 ± 0.09	4.82 ± 0.52	0.120 ± 0.010
	TP	0.53 ± 0.05	3.81 ± 0.15‡	0.138 ± 0.009
	EB	0.70 ± 0.05‡	4.75 ± 0.35	0.155 ± 0.013§
	Cast	0.73 ± 0.04‡	5.09 ± 0.05	0.143 ± 0.007§
	Cast + TP	0.60 ± 0.05	3.81 ± 0.09‡	0.157 ± 0.004§
Females	Intact	0.97 ± 0.05	5.39 ± 0.50	0.172 ± 0.020
	TP	0.73 ± 0.09§	4.42 ± 0.21§	0.165 ± 0.023
	EB	0.95 ± 0.08	5.54 ± 0.46	0.169 ± 0.013
	Cast	0.94 ± 0.04	4.99 ± 0.71	0.191 ± 0.018
	Cast + TP	0.73 ± 0.03§	4.44 ± 0.09§	0.165 ± 0.009

* Values are expressed as mean ± SD, N = 5.

† Sesame oil (Intact and Cast), testosterone propionate (TP, 50 mg/kg) and estradiol benzoate (EB, 1 mg/kg) were injected s.c., daily for 7 days. Cast means castration and ovariectomy.

‡ Significantly different ($P < 0.01$) from intact males.

§ Significantly different ($P < 0.01$) from intact females.

Table 2. Tissue TGSH levels in intact mice

	TGSH (mM)			
Sex	Kidney	Liver	Blood	Plasma
Males	4.55 ± 0.61	11.00 ± 0.96	0.97 ± 0.03	0.057 ± 0.004*
Females	4.36 ± 0.35	10.94 ± 0.93	0.87 ± 0.07	0.032 ± 0.005

Values are reported as mean ± SD, N = 7.8.

* Significantly different ($P < 0.01$) from females.

towards GSH and that the formation of MeHg–GSH complex is of critical importance for the renal accumulation of the administered MeHg [5, 6]. Experiments with BSO revealed that, although there was no significant difference in hepato-renal TGSH levels between the two sexes, the turnover rates of hepato-renal glutathione were significantly faster in males than in females. The current concept is that BSO is rapidly taken up by the liver and kidney and effectively inhibits *de novo* synthesis of GSH [16]. Since γ-glutamyltranspeptidase, the only known enzyme that hydrolyzes the γ-glutamyl linkage of GSH and its derivatives, localizes exclusively on the outer surface of plasma membranes [17], degradation of the tripeptide occurs extracellularly. Thus, under physiological conditions in which no electrophilic substrate for glutathione conjugation is challenged, the turnover rate and half-life of tissue TGSH have been assumed to reflect predominantly the rate of GSH efflux rather than the rate of its intracellular GSH utilization [14, 15]. Analysis of the castrated and hormone-treated animals revealed that, as observed with the metabolism of MeHg, the turnover of tissue TGSH was affected significantly by sex hormones. These observations suggest that the rate of MeHg handling *in vivo* may be closely correlated with the turnover rate of tissue TGSH. Consistent with this notion, both the turnover rate of tissue TGSH and the rate of urinary elimination of MeHg were significantly faster in males than in females. Furthermore, urinary excretion of Hg and the turnover of hepato-renal TGSH in females were accelerated by TP, whereas both processes were decreased by treating males with EB. It has been reported that treatment of animals with diethylmaleate which scavenges GSH reduces the hepatic GSH levels and decreases the renal accumulation of MeHg [18]. This fact is consistent with the notion described above.

It is known that TP treatment increases the tubular capacity to secrete organic anions without affecting renal plasma flow and glomerular filtration rate [19]. Recent studies [20, 21] revealed that GSH and related compounds including a mercapturic acid are secreted into urine by renal tubule cells. It is also known that MeHg accumulates in the kidney localized along the entire length of proximal tubules and that a secretory component predominates over filtration [22–24]. Thus, urinary occurrence of Hg might be closely correlated with the secretory function of the proximal tubules for GSH and its metabolites. Enhanced urinary excretion of Hg in TP-treated mice may also reflect, at least in part, the increased capacity of the kidney to secrete these metabolites into the lumenal compartment. Since enhanced urinary excretion of Hg in TP-treated males was found without change in the tissue turnover rates of TGSH, it seems that another renal

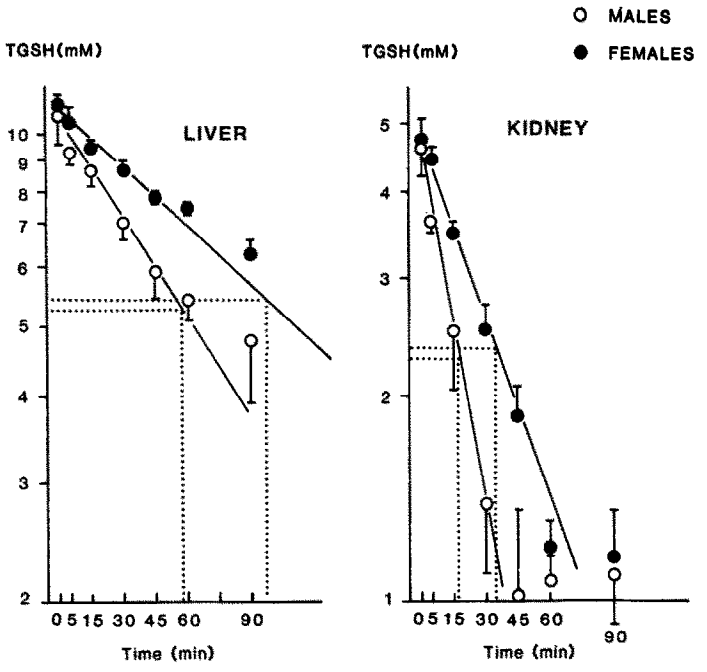

Fig. 3. Effect of buthionine sulfoximine on the hepato-renal TGSH levels in the intact males and females. Mice were injected with buthionine sulfoximine (via i.v. and i.p. at a dose of 1 mmol/kg respectively). At indicated intervals after the administration, hepato-renal TGSH levels were determined as described in the text. The data show the mean ± SD (N = 3).

Table 3. Effect of hormonal manipulation on renal and hepatic TGSH half-lives

	$T_{\frac{1}{2}}$* (min)			
	Kidney		Liver	
Treatment	Males	Females	Males	Females
Intact	18	35	57	100
Cast	37	38	90	95
TP	20	26	55	73
EB	36	37	95	96

* Half-life of TGSH was estimated from the semi-logarithmic plots of tisssue TGSH levels before and at 5, 15, 30, 45, 60 and 90 min after administration of buthionine sulfoximine. N = 3, for each point.

function may be concerned with renal excretion of MeHg in addition to turnover rate of tissue TGSH.

The present studies revealed that orally administered MeHg localizes in many tissues, such as the liver, brain, kidney and erythrocytes. Increased renal accumulation and urinary excretion of MeHg in TP-treated females was accompanied by concomitant decrease in its tissue levels. Thus, MeHg in these tissues may be transferred to the kidney for its urinary excretion. Since many cells and tissues including erythrocytes and liver have secretory transport system for GSH-adducts [7, 25], intracellular MeHg possibly may be secreted as its GS-conjugates. Alternatively, MeHg may come out of tissues via some thiol exchange mechanism in which redox states of blood and tissues play an important role. Among various tissues, the liver is the major organ which secretes glutathione in its reduced form into the circulation [7]; the excreted GSH circulates in all tissues, controls the redox state of these tissues, and is degraded predominantly in the kidney [7]. Thus, the marked difference in the rate of MeHg elimination in males and females may closely correlate with the difference in the rate of GSH turnover in the two sexes. The molecular form(s) of MeHg responsible for secretion across the membranes of various tissues as well as for interorgan transfer should be studied further.

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