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METABOLISM OF GLYCYRRHETIC ACID BY RAT LIVER MICROSOMES—III

MALE-SPECIFIC GLYCYRRHETINATE DEHYDROGENASE

TERUAKI AKAO,* TAIKO AKAO, MUNEO AOYAMA and KYOICHI KOBASHI
Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

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Abstract—Glycyrrhetinate (GA) dehydrogenase localized in microsomes of rat liver catalyses the oxidation and reverse reduction of 18β-glycyrrhetic acid (GA), an aglycone of glycyrrhizin and a main component of liquorice, to 3-keto-18β-glycyrrhetic acid (3-ketoGA). The enzyme activity was detected in microsomes of adult males, but not in those of adult females. It was not observed in infant males but appeared 6 weeks after birth, increased gradually and reached the maximum level at 12 weeks after birth, whereas it was not detected in the hepatic microsomes of females of any age. The administration of estradiol valerate to intact adult males decreased GA dehydrogenase activity remarkably. Castration of male rats also caused a marked reduction of the activity, but the administration of testosterone proprionate to these rats restored it to close to the normal level. On the other hand, ovariectomy of female rats did not bring the activity into existence, but the injection of testosterone proprionate to the ovariectomized rats brought it into a slight existence, in spite of no appearance of the activity by the treatment of testosterone proprionate to intact adult females. The sex-related difference in the activity in adults was eliminated by hypophysectomy of male and female rats, their microsomal activities after the operation being the same, 20–40% of the activity in intact males. Moreover, the administration of estradiol valerate to the hypophysectomized rats did not affect the activity. These results indicate that GA dehydrogenase is male-specific and regulated by sex-hormones through the pituitary.

Glycyrrhizin (GL†), a main component of liquorice (*Glycyrrhiza glabra* L.), is ingested orally as a sweetener or as a component in oriental medicine. GL has a steroid-like action [1] and an anti-viral activity [2]. When it is administered orally, 18β-glycyrrhetic acid (GA), an aglycone of GL, is detected in the sera of human subjects, but GL is not [3]. GL is metabolized to GA, and then 3-epi-18β-glycyrrhetic acid via 3-keto-18β-glycyrrhetic acid (3-ketoGA), by human intestinal bacteria [4–8], but not by the human liver [9]. GA shows pronounced anti-inflammatory activity [10, 11]. It thus appears that when GL is administered orally, it is hydrolysed by intestinal bacterial enzyme(s) to GA, which then exhibits the pharmacological activity. Carbenoxolone (3-*O*-β-carboxypropionyl-GA) administered to patients with gastric ulcers seems also to be hydrolysed to GA during the enterohepatic circulation, though it is absorbed mostly unchanged [12]. However, in human beings ingesting liquorice or administered carbenoxolone, pseudoaldosteronism is observed frequently [13–15]. Monder *et al.* [16] proposed that this side-effect induced with liquorice was due to inhibition of renal 11β-hydroxysteroid dehydrogenase by GA. Accordingly, it is important to clarify the metabolism of GA in animal tissues. However, studies on GA-metabolizing capacities of animal tissues are scarce, except those on glucuronidaton and sulfation of GA *in vivo* in rat followed by biliary excretion [17, 18]. Recently, we detected the reversible reaction between GA and 3-ketoGA catalysed by GA dehydrogenase [19] and the hydroxylation of GA and 3-ketoGA [20] in rat liver microsomes.

Many drug-metabolizing enzymes in rat livers show sex-related differentiation [21–23]. The present study reports such a differentiation in the activity of GA dehydrogenase in rat liver microsomes.

MATERIALS AND METHODS

Materials. GA was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 3-KetoGA was prepared as described previously [4]. Testosterone propionate, dexamethasone and phenobarbital sodium were from the Wako Pure Chemical Co. (Osaka, Japan). β-Estradiol 17-valerate and 20-methylcholanthrene were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). NADPH and $NADP^+$ were products of the Oriental Yeast Co. (Tokyo, Japan). All other reagents were of the best commercial quality available.

Animals and treatments. Wistar strains of male and female rats were used. Castration of male rats was performed at 4 weeks of age and ovariectomy of female rats was performed at 7 weeks of age. Male and female rats hypophysectomized and sham-operated at 4 weeks of age were purchased from Clea Japan (Tokyo, Japan). Testosterone propionate dissolved in olive oil was injected subcutaneously to intact females, castrated males and ovariectomized

* To whom correspondence should be addressed.

† Abbreviations: GA, 18β-glycyrrhetic acid (glycyrrhetinate); 3-ketoGA, 3-keto-18β-glycyrrhetic acid; GL, glycyrrhizin.

females at a dose of 10 mg/kg every other day for 10 days before being killed at 9–10 weeks of age. The same procedure was followed with the injection of estradiol valerate dissolved in olive oil to intact males and hypophysectomized rats. All control and sham-operated rats were treated with olive oil only.

Dexamethasone suspended in olive oil was given orally to male and female rats at the dose of 300 mg/kg/day for 4 days before being killed at 11 weeks of age. Some male rats were treated intraperitoneally for 4 days with phenobarbital sodium dissolved in saline at the dose of 80 mg/kg/day or with 20-methylcholanthrene dissolved in olive oil at the dose of 40 mg/kg/day before being killed at 8 weeks of age. Control rats were treated at the same time with olive oil or saline only.

Preparation of microsomes. Liver microsomes were prepared as reported by Kuriyama *et al.* [24], except that 0.15 M KCl was used for the homogenization of perfused livers instead of 0.25 M sucrose, and washed with 0.15 M KCl containing 10 mM ethylenediaminetetraacetic acid.

Thin-layer chromatography (TLC). TLC for GA and 3-ketoGA was performed on silica gel plates (Merck, silica gel 60F-254, layer thickness 0.25 mm) with a solvent system of chloroform/petroleum ether/acetic acid (5:5:1 by vol.). The quantity was analysed with a TLC scanner as described previously [7].

Enzyme assay. The enzyme activities for oxidation of GA and reduction of 3-ketoGA were measured as described previously [19]. The assay mixture for oxidation contained 50 nmol of GA, 1 μmol of $NADP^+$, microsomes (200–1000 μg protein) and 0.1 M potassium phosphate buffer (pH 8.0) in a final volume of 0.5 mL. The assay mixture for reduction contained 50 nmol of 3-ketoGA, 1 μmol of NADPH, microsomes (100–400 μg protein) and 0.1 M potassium phosphate buffer (pH 7.2) in a final volume of 0.5 mL. The mixture was incubated at 37° for 10–30 min, and the reaction was stopped by the addition of 1 N HCl. The mixture was extracted twice with 2 mL of ethyl acetate and, after drying, the residue of the ethyl acetate layer was analysed by TLC, as described above.

Determination of protein. Protein was determined by the method of Lowry *et al.* [25] using bovine serum albumin as the standard.

RESULTS

Sex difference and developmental change in GA dehydrogenase

The oxidation and the reverse reduction of GA to 3-ketoGA, catalysed by GA dehydrogenase, were observed in hepatic microsomes of adult male rats. However, they were not detected in those of adult female rats at all, even though the reaction time was prolonged to more than 2 hr and a large amount of hepatic microsomes from the female was added to the reaction mixture.

As shown in Fig. 1, developmental appearance of microsomal GA dehydrogenase activity was observed in male rats. The microsomal activity was not detected at 4 weeks after birth, but appeared 6 weeks after birth and increased gradually. The activity reached a maximal level at 12 weeks of age, keeping at this level for more than 6 months. In contrast, no activity was observed in hepatic microsomes of female rats of any age.

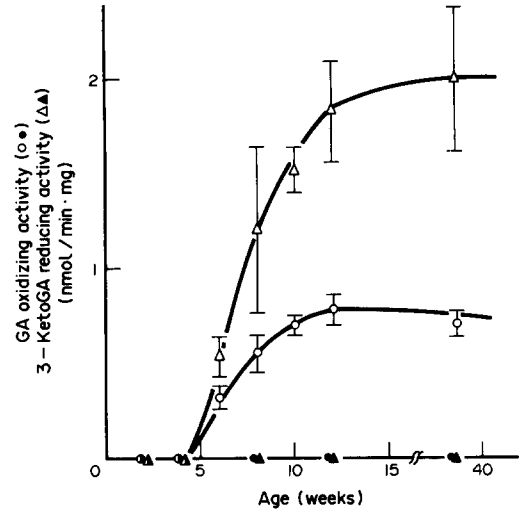

Fig. 1. Postnatal development of GA dehydrogenase activity in liver microsomes from male (○, △) and female (●, ▲) rats. Vertical bars represent the standard deviation of the average obtained from six rats at 8 weeks of age and three rats in all other cases.

Effects of sex hormones

Injection of estradiol valerate to adult male rats caused a remarkable decrease of microsomal GA dehydrogenase activity (Table 1). However, injection of testosterone propionate to adult female rats did not result in an appearance of the activity. As shown in Table 2, castration of male rats at 4 weeks of age caused a decrease of the enzyme activity at 9 weeks of age to about 30% activity in sham-operated rats of the same age. Treatment of castrated rats with testosterone propionate at 8 weeks of age restored the activity to close to the normal level. In the case of castration at 7 weeks of age similar results were also observed (data not shown). Ovariectomy of female rats had no effect on GA dehydrogenase activity, but treatment of ovariectomized rats with testosterone propionate brought the microsomal activity into a slight existence (Table 2), in spite of no appearance of the activity by the treatment of intact female rats with testosterone propionate (Table 1). These results indicate that the synthesis of GA dehydrogenase is regulated by sex hormones.

Regulation through pituitary gland

In recent studies on the hormonal regulation of sexually differentiated cytochromes P450, testosterone and estradiol have been shown to affect the expression of the cytochromes by the regulation of serum levels of growth hormone through the hypothalamus–pituitary system [26–29]. To verify the influence of the pituitary gland on hepatic GA dehydrogenase activity, male and female rats were hypophysectomized. Hypophysectomy of male rats

Table 1. Effects of estradiol and testosterone treatments on GA dehydrogenase activity in liver microsomes of male and female rats

Administration*	GA dehydrogenase activities† (nmol/min · mg) GA-oxidizing	3-ketoGA-reducing
Male		
—	0.52 ± 0.16	1.36 ± 0.30
Estradiol	0.074 ± 0.016	0.17 ± 0.04
Female		
—	ND	ND
Testosterone	ND	ND

* Estradiol valerate and testosterone propionate dissolved in olive oil were administered subcutaneously every other day for 10 days to one group of four male and female rats, respectively. Control rats received olive oil only. The rats were killed at 10 weeks of age.
† Means ± SD.
ND, not detected.

Table 2. Effects of castration, ovariectomy and testosterone administration on GA dehydrogenase activity in liver microsomes of male and female rats

Treatment*	GA dehydrogenase activities† (nmol/min · mg) GA-oxidizing	3-ketoGA-reducing
Male		
Sham	0.57 ± 0.12	1.20 ± 0.37
Castration	0.17 ± 0.07	0.43 ± 0.24
Castration + testosterone	0.40 ± 0.23	0.93 ± 0.50
Female		
Sham	ND	ND
Ovariectomy	ND	ND
Ovariectomy + testosterone	0.051 ± 0.043	0.071 ± 0.057

* Male rats were castrated at 4 weeks of age. Female rats were ovariectomized at 7 weeks of age. Two groups of four castrated and three ovariectomized rats were injected subcutaneously with testosterone propionate every other day for 10 days. Other groups of four castrated, four sham-operated male, three ovariectomized and three sham-operated female rats received olive oil only. Male and female rats were killed at 9 weeks and 10 weeks of age, respectively.
† Means ± SD.
ND, not detected.

caused a marked decrease in the activity and that of female rats caused a considerable increase in the activity to 20–30% activity of the control males, though sham-operation of male and female rats did not affect the activity (Table 3). Thus, the sex difference of the enzyme activity was abolished by hypophysectomy.

Injection of estradiol valerate to hypophysectomized rats of both sexes had no effect on the activity (Table 3), in spite of the remarkable decrease in the activity of intact males caused by this treatment (Table 1). These results suggest that the synthesis of GA dehydrogenase is regulated by sex hormones through the hypothalamus–pituitary system.

Effects of inducers of cytochromes P450

Some of the cytochromes P450 which are sex differentiated and regulated by the hypothalamus–pituitary system are induced with phenobarbital or steroid derivatives such as dexamethasone and pregnenolone-16β-carbonitrile [29]. Injection of phenobarbital or dexamethasone to rats resulted in a considerable decrease of GA dehydrogenase activity to the extent of 30% or 45% of the normal level, respectively (Table 4), despite the increase in content of microsomal cytochromes P450. Treatment with 20-methylcholanthrene, another type of cytochrome P450 inducer, did not affect the activity.

DISCUSSION

Male-specific developmental appearance (Fig. 1) of GA dehydrogenase and sex hormone-dependent regulation of its synthesis through the hypothalamus–pituitary system (Tables 1–3) are the same phenomena as associated with cytochrome P450h(male) [26, 27, 30, 31]. Also, the decrease in GA dehydrogenase activity caused by the treatment

Table 3. Effects of hypophysectomy and estradiol administration on GA dehydrogenase activity in liver microsomes of male and female rats

Treatment*		GA dehydrogenase activities† (nmol/min · mg) GA-oxidizing	3-ketoGA-reducing
Sham-operated	Male	0.67 ± 0.14	1.92 ± 0.32
	Female	ND	ND
Hypophysectomy	Male	0.20 ± 0.04	0.32 ± 0.08
	Female	0.15 ± 0.05	0.23 ± 0.05
Hypophysectomy +estradiol	Male	0.23 ± 0.04	0.45 ± 0.04
	Female	0.18 ± 0.01	0.41 ± 0.05

* Male and female rats were hypophysectomized and sham-operated at 4 weeks of age. One hypophysectomized group of three male and three female rats was injected subcutaneously with estradiol valerate every other day for 10 days before being killed. Control hypophysectomized group of 3 male and 3 female rats was administered with olive oil only. The hypophysectomized and sham-operated rats were killed at 9 weeks and 10 weeks of age, respectively.

† Means ± SD.

ND, not detected.

Table 4. Effects of phenobarbital, 20-methylcholanthrene and dexamethasone treatments on GA dehydrogenase activity in liver microsomes of male rats

Administration*	GA dehydrogenase activities† (nmol/min · mg) GA-oxidizing	3-ketoGA-reducing
No treatment	0.55 ± 0.02	1.20 ± 0.44
Phenobarbital	0.21 ± 0.11	0.40 ± 0.13
20-Methylcholanthrene	0.52 ± 0.03	1.25 ± 0.38
Dexamethasone	0.34 ± 0.10	0.76 ± 0.04
Olive oil	0.82 ± 0.07	2.00 ± 0.01

* Two groups of three rats were injected intraperitorially with phenobarbital in saline and 20-methylcholanthrene in olive oil, respectively, daily for 4 days. Control and treated rats were killed at 8 weeks of age. Two groups of three rats were administered orally with dexamethasone in olive oil and olive oil only, respectively, daily for 4 days. The rats were killed at 11 weeks of age.

† Means ± SD.

of rats with xenobiotics such as phenobarbital and dexamethasone (Table 4) resembles the decrease of cytochrome P450h(2c) with many xenobiotics [32]. Accordingly, the expression of GA dehydrogenase in rat liver seems to be regulated in the same way as cytochrome P450h.

Although sex-differences in many enzyme activities, related to drug and steroid metabolism in rat liver, are known [21–23, 27, 33, 34], a strict sex-difference in GA dehydrogenase activity, which is not detected in female microsomes at all, has not been observed before. Moreover, various sex-differentiated hydroxysteroid dehydrogenase and testosterone hydroxylase activities in microsomes were detectable either in male or in female, and these activities seem to be exhibited by more than two enzymes [35]. In addition, the measurement of testosterone hydroxylase activity is difficult owing to the high activity of 5α-reductase in mature female microsomes. It seems that GA dehydrogenase is recognized as a suitable marker indicating a sex-related difference in rat liver. GA is also hydroxylated in rat liver microsomes [20] and a sex-related difference in this activity is also observed (unpublished data). Horwich and Galloway [14] reported that carbenoxolone treatment caused oedema of the ankles and pretibial area in women, but in man there was a greater tendency to hypertension and oedema seldom developed. A sex-related difference in GA metabolism is of interest in the case of GL- and carbenoxolone-induced pseudoaldosteronism, as it has been proposed that the syndrome is due to inhibition of renal 11β-hydroxysteroid dehydrogenase by GA [16].

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