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DISCONTINUOUS VARIATION IN HEPATIC URIDINE DIPHOSPHATE GLUCURONYLTRANSFERASE TOWARD ANDROSTERONE IN WISTAR RATS

A REGULATORY FACTOR FOR *IN VIVO* METABOLISM OF ANDROSTERONE

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Abstract—Male and female Wistar rats showed discontinuous variation in hepatic microsomal UDP-glucuronyltransferase activities toward androsterone but not toward testosterone. In contrast, Sprague–Dawley rats, Hartley guinea pigs and CF#1 mice did not exhibit such diversity. Microsomes with a high level of transferase activity toward androsterone (HG) and microsomes with a low level of transferase activity (LG) were found approximately in the ratio 5:4 in male and female Wistar rats, the HG to LG specific activity ratio being about 9. In the presence of Triton X-100, the enzyme activity was stimulated maximally (5-fold) and the HG to LG specific activity ratio increased to 16. Kinetic studies revealed that the transferase had a much lower affinity toward androsterone in the LG microsomes than in the HG microsomes. These results confirm the view that hepatic UDP-glucuronyltransferase is principally responsible for the large variations in the metabolism *in vivo* of androsterone and testosterone in female Wistar rats.

Glucuronidation plays an important role in the detoxication of steroid hormones. Hepatic microsomal UDP-glucuronyltransferase (EC 2.4.1.17) catalyzes the transfer of glucuronic acid from UDP-glucuronic acid to various steroids. Considerable evidence, from studies with several steroid substrates [1–6], has been accumulated to demonstrate the multiplicity of action of UDP-glucuronyltransferase. However, the mechanisms responsible for their regulation remain unclarified.

Recent studies from this laboratory showed marked variations in biliary metabolites of androsterone [7] and testosterone [8] in female Wistar rats. Additional studies with androsterone glucuronide and androsterone sulfate suggest that hepatic UDP-glucuronyltransferase may be principally responsible for large variations in the metabolism *in vivo* of androsterone and testosterone in female rats [9].

The present study was designed to compare the glucuronidation of androsterone and testosterone by liver microsomes from Wistar rats and other animals. The study includes effects of Triton X-100 on the enzyme activity and determination of kinetic parameters.

MATERIALS AND METHODS

Materials. [1,2-^{3}H]androsterone (44.5 Ci/m-mol) and [4-^{14}C]testosterone (20.2 mCi/m-mol) were purchased from New England Nuclear, Boston, Ma, U.S.A. Preparation of [1,2-^{3}H]androsterone glucuronide (8.6 μCi/μmol) and unlabeled androsterone glucuronide was described previously [9]. The radiochemical purities of these labeled steroids were confirmed by thin-layer chromatography (t.l.c.) shortly before use, as described elsewhere [7–9], and were more than 98 per cent. Androsterone and testosterone were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. UDP-glucuronic acid disodium salt and β-glucuronidase (Ketodase) were purchased from Boehringer, Mannheim, West Germany, and Warner-Chilcott, Morris Plains, NJ, U.S.A. respectively. All other reagents were of analytical grade.

Animals and preparation of microsomal fractions. Male and female rats of the Wistar strain (160–240 g: Matsumoto Experimental Animal Lab., Tokyo, Japan) were used throughout this study. For the specific activity study, the following adult male animals were obtained from Matsumoto Experimental Animal Lab., Tokyo, Japan: Sprague–Dawley rats (215–230 g), Hartley guinea pigs (175–190 g) and CF#1 mice (20 g). The animals had free access to food and water, were decapitated and a 20% (w/v) liver homogenate was prepared in ice-cold 0.25 M sucrose in 0.1 M Tris–HCl buffer, pH 7.2, with a Teflon–glass homogenizer. Microsomal fractions were collected by differential centrifugation (2000 *g* for 10 min, 16,000 *g* for 45 min, and 105,000 *g* for 60 min) in a Hitachi 65P ultracentrifuge. The microsomal pellets were resuspended in 0.25 M sucrose in 0.1 M Tris–HCl buffer, pH 7.2. All procedures for the isolation of microsomal fractions were done at 0–4°. The microsomal protein was estimated by the method of Lowry *et al.* [10], using bovine serum albumin as the protein standard.

Enzyme assays. UDP-glucuronyltransferase activity was assayed by a modification of the method described by Rao *et al.* [11]. The ethanolic solution of the

substrate steroid was evaporated gently under N_2 and the residue was dissolved in one drop of propylene glycol. The standard incubation medium contained microsomal fraction (1.5 to 2.4 mg protein), 0.1 M Tris–HCl buffer, pH 7.2, 20 μM EDTA, 10 mM $MgCl_2$, 2 mM UDP-glucuronic acid and 172 μM [^{3}H]androsterone (0.091 μCi) or 692 μM [^{14}C]testosterone (0.045 μCi) in a total volume of 2.0 ml. The standard incubation was carried out for 20 min at 37° and terminated by heating in a boiling water-bath for 10 min, followed by cooling in ice-water. The blank values were obtained from control incubations in which UDP-glucuronic acid was omitted. For the assay of activated transferase activity, 0.05% (w/v) Triton X-100 [12] was added to the incubation medium in which UDP-glucuronic acid was omitted. The mixture was then preincubated for 30 min at 4°, the reaction was started by the addition of UDP-glucuronic acid, and the incubation was allowed to proceed at 37° for 20 min. For kinetic studies, the assay was identical with the standard incubation except that different sets of substrate concentrations were employed: [^{3}H]androsterone (17.2 to 344 μM, 0.091 μCi) or [^{14}C]testosterone (34.6 to 692 μM, 0.045 μCi) with a fixed concentration of UDP-glucuronic acid (2 mM) for the determination of K_m and V_{max} values for steroids; UDP-glucuronic acid (50–1200 μM) with a fixed concentration of [^{3}H androsterone (172 μM, 0.091 μCi) for the determination of K_m for UDP-glucuronic acid. For the inhibitory experiments, variable concentrations of [^{3}H] androsterone (17.2 to 172 μM, 0.091 μCi) and a fixed concentration of UDP-glucuronic acid (2 mM) were incubated in the presence or absence of 173 μM testosterone. After incubation, the mixture was diluted with 30 ml water and extracted twice with 60 ml ethyl acetate. The ethyl acetate extracts were combined, washed with water, dried over Na_2SO_4, and evaporated *in vacuo* to give the free steroid fraction. The combined aqueous phase was evaporated *in vacuo* to afford the glucuronide fraction. The free steroid and glucuronide fractions were stored as 10 (or 25) ml of ethanol and aqueous solutions, respectively, and a 0.5-ml portion of these fractions was submitted for the measurement of the radioactivity. The rate of glucuronidation was corrected by the blank value.

Identification of the enzymatic products. The steroids obtained by incubating [^{3}H]androsterone or [^{14}C] testosterone with various liver microsomes from male and female Wistar rats were worked up separately. The glucuronide fraction was separated by column chromatography on XAD-2 resin and subsequently hydrolyzed by incubation with β-glucuronidase to give the liberated steroid fraction as described previously [13]. The free and liberated steroid fractions were separated by t.l.c. on silica gel GF (Merck) using chloroform–acetone (29:1, v/v) as solvent. The radioactive zones were detected with a Packard model 7201 autoscanner, scraped off, and eluted with methanol. The separated steroids were trimethylsilylated [14] and analyzed by gas chromatography on a Shimadzu GC-4BM chromatograph with a flame ionization detector using 0.5% CHDMS and 1.5% SE-30 as the stationary phase as described previously [7]. Relative retention times were calculated relative to 5α-cholestane.

Radioactivity measurements. Radioactivity was counted in an Aloka LSC-502 liquid scintillation spectrometer in a toluene medium as described previously [13]. Efficiencies of ^{3}H and ^{14}C counting were about 40 and 80 per cent respectively. Results are expressed in dis/min.

RESULTS

Glucuronidation of androsterone and testosterone by Wistar rat liver microsomes. After incubation of [^{3}H]androsterone and [^{14}C]testosterone with male and female Wistar rat liver microsomes, the total recoveries of the ^{3}H and ^{14}C radioactivities in the free steroid and glucuronide fractions were quantitative. The rates of glucuronidation of androsterone and testosterone were linear with respect to time for 40 min and linear with respect to the addition of microsomal protein up to 5 mg. By varying the substrate concentrations, the reaction rates reached plateaux at about 100 and 500 μM by incubation with androsterone and testosterone respectively. Rao *et al.* [3] reported that testosterone was not completely soluble in the assay medium when concentrations were above 700 μM. The incubation conditions were established based on these observations. The transferase activity is latent and the maximal activation of the enzyme is achieved by treatment of liver microsomes with detergents such as 0.05–0.10% (w/v) Triton X-100 [6, 12, 15]. By pretreatment with 0.05% (w/v) Triton X-100, the enzyme activity was stimulated maximally (5-fold).

After β-glucuronidase hydrolysis of the glucuronide fraction, approximately 96 per cent of the ^{3}H and ^{14}C appeared in the liberated steroid fraction. Examination of the free and liberated steroid fractions by t.l.c. showed the presence of a single radioactive peak corresponding to the respective substrate steroid. Gas chromatography of the trimethylsilyl derivatives on CHDMS (SE-30) afforded relative retention times of 0.75 (0.39) or 2.09 (0.65) for both free and liberated steroid fractions obtained by incubation with androsterone or testosterone respectively. These relative retention times were identical with those of the respective substrate steroids. These results show that glucuronidation was the sole biochemical reaction which occurred during incubation and confirm the validity of this assay method.

Kinetic studies on the glucuronidation of androsterone and testosterone by Wistar rat liver microsomes. The specific activities of UDP-glucuronyltransferase toward androsterone and testosterone are listed in Table 1. With androsterone or testosterone as substrate, the transferase activities from females were, on an average, 2.1- or 1.5-fold higher than those from males respectively. A pronounced feature is the existence of discontinuous variation in the transferase activity toward androsterone, irrespective of the sex. Rats could be divided into three groups based on the enzyme activity. Approximately 50 per cent of the microsomes (HG) gave high specific activity, whereas about 40 per cent of the microsomes (LG) afforded very low activity. The HG to LG specific activity ratio was about 9. Less than 10 per cent of the microsomes were intermediate between the HG and LG microsomes. In contrast to this, no appreciable

Table 1. Hepatic UDP-glucuronyltransferase activity toward androsterone and testosterone in Wistar rats

Sex	Microsomes*	Treatment†	UDP-glucuronyltransferase activity	
			Androsterone	Testosterone
Female	HG	Native	657 ± 176‡ (9)	1190 ± 296 (9)
		Activated	2070 ± 436 (7)	3520 ± 470 (7)
	Intermediate	Native	267 (1)	851 (1)
		Activated	1140 (1)	2210 (1)
	LG	Native	77 ± 26(6)	994 ± 361 (6)
		Activated	123 ± 37 (4)	3050 ± 615 (4)
Male	HG	Native	289 ± 30 (6)	682 ± 253 (6)
		Activated	1480 ± 261 (4)	2640 ± 476 (4)
	Intermediate	Native	106 (1)	1000 (1)
		Activated	442 (1)	2300 (1)
	LG	Native	31 ± 11 (5)	539 ± 149 (5)
		Activated	97 ± 25 (3)	2120 ± 167 (3)

*HG: microsomes with a high specific activity toward androsterone; LG: microsomes with a low specific activity toward androsterone

†Native: native microsomes; activated: microsomes activated with Triton X-100.

‡Mean ± S. D. (number of animals). The enzyme activity is expressed as pmol glucuronide/min/mg of protein.

variation was observed in the rate of glucuronide formation with testosterone among these groups. The marked diversity of androsterone glucuronidation was amplified by the activation of the transferase with Triton X-100 as shown in Table 1. The detergent stimulated androsterone glucuronidation 3- to 5- and 2- to 3-fold in the HG and LG microsomes, respectively. Thus, the HG to LG specific activity ratio increased from 9 to 16 by the detergent. Treatment with the detergent increased the enzyme activity toward testosterone by 3- to 4-fold among these groups.

Incubation of 340 μM [^{3}H]androsterone glucuronide (0.068 μCi) with the assay medium containing the LG microsomes resulted in 98 per cent recovery of androsterone glucuronide from the assay medium.

The apparent K_m and V_{max} values for androsterone and testosterone were determined by Lineweaver–Burk plots and are shown in Table 2. With androsterone and testosterone as substrates, male and female rats provided similar K_m values for the respective HG and LH groups. In general, the apparent K_m values with Triton X-100-treated microsomes were about 1.3-fold higher than those of the native microsomes. The distinctive feature is that the LG microsomes exhibited about a 4-fold higher K_m for androsterone as compared with the HG microsomes, whereas the LG microsomes gave only a 1.3-fold higher K_m for testosterone than the HG microsomes. On the other hand, measurement of K_m for UDP-glucuronic acid at a fixed concentration of androsterone revealed that the apparent K_m values for UDP-glucuronic acid were quite similar between the HG and LG microsomes. The apparent K_m values were 417 and 357 μM in the female HG and LG microsomes respectively. The corresponding figures for

Table 2. Apparent kinetic parameters of hepatic UDP-glucuronyltransferase toward androsterone and testosterone in Wistar rats

Sex	Microsomes*	Treatment†	Androsterone		Testosterone	
			K_m (μM)	V_{max}‡	K_m (μM)	V_{max}
Female	HG	Native	20§	1440	133	2240
		Activated	33	2970	192	5060
	LG	Native	100	170	172	3700
		Activated	100	179	227	5170
Male	HG	Native	24	636	100	1400
		Activated	31	2140	167	3660
	LG	Native	97	127	143	1350
		Activated	118	284	200	3060

*HG: microsomes with a high specific activity toward androsterone; LG: microsomes with a low specific activity toward androsterone

†Native: native microsomes; activated: microsomes activated with Triton X-100

‡V_{max} is expressed as pmol glucuronide/min/mg of protein.

§Each value represents the mean of two animals.

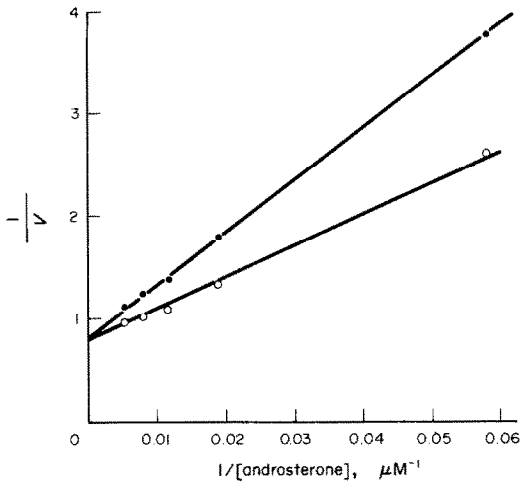

Fig. 1. Double reciprocal plot of initial velocity against variable androsteone concentrations at 2 mM UDP-glucuronic acid in the female Wistar rat liver microsomes with a high level of UDP-glucuronyltransferase toward androsterone as described in Materials and Methods, in the presence (●) or absence (○) of 173 μM tetosterone. Activities are expressed as nmol androsterone glucuronidated/min/mg of protein.

males were 333 and 385 μM respectively. As expected, the V_{max} values for androsterone and testosterone paralleled the specific activities described above. When androsterone glucuronidation was assayed with the HG microsomes in the presence or absence of testosterone and double-reciprocal plots were made of the data, the results indicated that testosterone competitively inhibited androsterone conjugation, with an apparent K_i of 205 μM for the female (Fig. 1) and a K_i of 112 μM for the male.

Rates of glucuronidation of androsterone and testosterone with male Sprague–Dawley rats, Hartley guinea pigs and CF#1 mice. In order to study strain and species differences, the specific activities of UDP-glucuronyltransferase toward androsterone and testosterone were measured by incubation with microsomes obtained from male Sprague–Dawley rats, Hartley guinea pigs and CF#1 mice and are shown in Table 3. Sprague–Dawley rats showed the highest transferase activity toward androsterone and testosterone, while CF#1 mice gave the lowest activity toward two substrates. In contrast to Wistar rats, these animals did not show marked diversity in the specific activity toward androsterone as well as testosterone.

Table 3. Hepatic UDP-glucuronyltransferase activity toward androsterone and testosterone in male Sprague–Dawley rats, Hartley guinea pigs and CF#1 mice*

	UDP-glucuronyltransferase activity	
Animal	Androsterone	Testosterone
Sprague–Dawley rat	911 ± 298	1140 ± 361
Hartley guinea pig	481 ± 174	646 ± 212
CF#1 mouse	118 ± 32	198 ± 34

*Enzyme activity is expressed as pmol glucuronide/min/mg of protein. Each value represents the mean ± S. D. of five animals.

DISCUSSION

The present study demonstrates that male and female Wistar rats give discontinuous variation in hepatic microsomal UDP-glucuronyltransferase activities toward androsterone but not toward testosterone. The rats with a high level of transferase activity toward androsterone (HG) and the rats with a low level of transferase activity (LG) were found approximately in the ratio 5:4. In contrast, Sprague–Dawley rats, Hartley guinea pigs and CF#1 mice did not show such diversity. It is of interest to note the existence of strain differences between Wistar and Sprague–Dawley rats.

The microsomal UDP-glucuronyltransferase is latent, probably buried inside the microsomal vesicle and can be activated by physical, chemical or enzymatic perturbation of the membrane structure [16, 17]. Triton X-100 is one of the typical xenobiotic activators. However, activation of the transferase with Triton X-100 rather amplified the diversity between HG and LG microsomes.

The observed low activity of the LG microsomes could not be ascribable to a high level of microsomal UDP-glucuronic acid pyrophosphatase which hydrolyzes UDP-glucuronic acid, because the LG microsomes formed testosterone glucuronide similar to the extent of the HG microsomes. Quantitative recovery of the incubated androsterone glucuronide from the assay medium containing the LG microsomes indicates that β-glucuronidase, the other microsomal enzyme which may affect the glucuronidation, was not involved in the hydrolysis of androsterone glucuronide formed in the assay medium.

Though kinetic data may lead to multiple interpretations with the restricted membrane-bound enzyme and nonpolar substrates [17, 18], comparison of the apparent K_m and V_{max} values obtained from native and activated microsomes revealed several interesting features. With androsterone and testosterone as substrates, male and female rats gave similar K_m values for the respective HG or LG microsomes, while the V_{max} exhibited sex differences, with females showing greater enzyme activity than males. The K_m values were slightly increased by detergent treatment, although marked increases in V_{max} were observed except for the V_{max} of androsterone in female LG microsomes. The results indicate that the affinity of the transferase toward androsterone and testosterone was not appreciably affected by the detergent and that the detergent might change the microsomal membranes, by which means the formerly latent enzyme may become accessible to the substrate leading to an increase of the velocity. The most striking aspect is that the apparent K_m for androsterone was much higher in the LG microsomes than in the HG microsomes, whereas the apparent K_m values for UDP-glucuronic acid were quite similar between the HG and LG groups. On the other hand, the apparent K_m values for testosterone were slightly higher in the LG microsomes than in the HG microsomes. These results imply that the low affinity of UDP-glucuronyl-

transferase toward androsterone must be responsible for the poor glucuronidation of androsterone in the LG microsomes. The low transferase activity of the LG microsomes can be interpreted as a defective transferase toward androsterone and/or abnormal microsomal membrane microenvironment of the transferase [17, 18]. The different kinetic behaviours of the transferase toward androsterone and testosterone in the HG and LG microsomes suggest the existence of multiple steroid UDP-glucuronyltransferases. However, the apparent multiplicity of the transferase may be caused by the existence of a single enzyme in several different lipid-stabilized conformations. Zakim and Vessey [16, 19] suggest that the microsomal lipids may affect the activity of UDP-glucuronyltransferase by concentrating the substrate and restricting the orientation of the substrate at the active site of the enzyme. We demonstrated that the transferase toward androsterone was competitively inhibited by testosterone in the HG microsomes. However, this does not necessarily mean that androsterone and testosterone can be glucuronidated at the same enzymatic active site. It is difficult to distinguish between these mechanisms from the present study. Purification of the transferase is necessary before its heterogeneity can be established.

The glucuronidation *in vivo* can be affected by a number of factors such as regulation of UDP-glucuronyltransferase, availability of UDP-glucuronic acid, hydrolysis of the glucuronide by β-glucuronidase, and availability of other metabolizing enzymes [17, 20]. The relationship between the transferase activity observed *in vitro* and the rate of glucuronidation in intact animals is not clear. However, the comparison studies on glucuronidation rate in intact organ, slice, cell and broken-cell preparations seem to indicate that UDP-glucuronyltransferase *in vivo* appears to be neither fully activated nor fully latent and that the transferase may be operating largely in a constrained form in the cell [12, 17]. In previous papers, we reported the existence of marked variations in biliary metabolites of androsterone [7] and testosterone [8] in female Wistar rats. It was found that about half of the rats excreted steroid metabolites very rapidly into bile (HE rats), whereas the remaining rats excreted the metabolites at a much slower rate (LE rats). The major metabolic pathway of androsterone and testosterone in HE rats was via formation of androsterone glucuronide, while the main pathway in LE rats was via formation of the monosulfates of $C_{19}O_2$ and $C_{19}O_3$ steroids. Additional studies revealed that the injected androsterone glucuronide was excreted rapidly and almost entirely in the unchanged form in the bile, while the injected androsterone sulfate gave mainly the mono- and disulfates of $C_{19}O_2$ and $C_{19}O_3$ steroids [9]. The present results, together with the previous findings, indicate that the discontinuous variation of UDP-glucuronyltransferase activity toward androsterone should be principally responsible for the large variations in the metabolism *in vivo* of androsterone and testosterone in female Wistar rats. Therefore, the following metabolic pathway of androsterone and testosterone could be reasonably formulated. Androsterone administered or biotransformed from testosterone must be mainly conjugated with glucuronic acid in the HE rat, due to a high level of UDP-glucuronyltransferase toward androsterone, and excreted rapidly in the bile. In the LE rat, a low level of the transferase toward androsterone should lead to the impaired glucuronide formation and predominant production of sulfuric acid conjugates.

The hereditary deficiency of UDP-glucuronyltransferase toward bilirubin and several substrates is well known in Gunn rats, the mutant strain of Wistar rats [17, 20]. In humans, Crigler–Najjar syndrome and Gilbert's disease are characterized by a genetically determined defect or insufficiency of bilirubin glucuronidation [21]. Our colony of Wistar rats may be another example of UDP-glucuronyltransferase insufficiency of genetic origin. Further studies with this experimental model system may be of interest for obtaining insight into the regulatory mechanism and heterogeneity of UDP-glucuronyltransferase

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