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CHARACTERISTICS OF NEONATAL ANDROGEN-INDUCED IMPRINTING OF RAT HEPATIC MICROSOMAL MONOOXYGENASES*

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Abstract—Both the apparent K_m of hepatic microsomal ethylmorphine *N*-demethylase and the responsiveness of this enzyme to testosterone treatment in adult rats are imprinted neonatally by androgen. The maintenance of these imprinted male-specific characteristics of this enzyme activity depends on the presence of an intact pituitary. Hypophysectomy alters the apparent K_m of this enzyme activity from male into female type, whereas castration, thyroparathyroidectomy or adrenalectomy does not. Androgen or estrogen administration has opposite effects on the apparent K_m, but similar effects on plasma FSH and LH concentrations. These results tend to mitigate against mediator roles for ACTH, TSH, LH and FSH.

The sensitive periods for androgen-induced imprinting of the apparent K_m of ethylmorphine *N*-demethylase differs from that of the responsiveness of this enzyme. With the former, imprinting occurs at days 2 and 4 of age, whereas the latter extended beyond days 2 and 4 to days 12 and 14 of age. Unlike imprinting in the CNS, neonatally administered estradiol-17β is ineffective in inducing the male characteristics of this enzyme activity.

Although hypophysectomy alters the imprinted male characteristics of the ethylmorphine *N*-demethylase (apparent K_m and responsiveness) to the female type, it shifts the neonatal androgen-imprinted differences in the turnover of cytochrome P-450 from female toward that of the male type. These results suggest that different hypophyseal factors may be responsible for the regulation of these neonatal androgen-imprinted male characteristics in hepatic microsomal monooxygenases.

Certain sex-dependent differences in the oxidative metabolism of aminopyrine, ethylmorphine, hexobarbital [1–3], and endogenous steroids [4–6] are known to occur in adult rats. Some aspects of these differences are modulated by the plasma concentration of testosterone. Castration of a male or testosterone treatment of a female, therefore, results in respective decreases or enhancements of drug-metabolizing enzyme activities in these animals. Another aspect of the sex-dependent differences, however, appears to relate to the imprinting influence of androgen during the early postnatal life. For example, the development of certain sex-dependent differences in the metabolism of corticosteroid and testosterone by the hepatic microsomal systems [7–9], the apparent K_m as well as the responsiveness of hepatic microsomal ethylmorphine *N*-demethylase [10], and the development of hepatic cytochrome P-450 of different turnover forms [11] have been documented as determined neonatally by testicular androgen.

To learn more about the neonatal androgen-induced imprinting of the hepatic microsomal monooxygenases in adult male rats, we have measured the apparent K_m and responsiveness of ethylmorphine *N*-demethylase as the end point. An assessment was made to determine the critical period when these male-specific characters are being imprinted or programmed. Neonatal testicular androgen(s) has long been recognized as an inducer(s) for the differentiation of neural tissues which determine the gonadotropin release pattern and the behavior in the male [12]. Attempts were made in this study to compare the steroid specificity between the imprinting of neural as against the hepatic microsomal monooxygenases. Evidence is also presented to suggest that different hypophyseal factor(s) may be required to maintain the male-specific characteristics of hepatic monooxygenases, e.g. the apparent K_m, the responsiveness of ethylmorphine *N*-demethylase and the turnover of different forms of hepatic cytochrome P-450.

MATERIALS AND METHODS

Animals and surgical procedures. Albino rats of Wistar Strain were purchased from Canadian Breeding Farm (St. Constant, Quebec) and maintained on Purina laboratory chow and water *ad lib.* Rats adrenalectomized, thyroparathyroidectomized or hypophysectomized as adult were supplied with NaCl (0.9% w/w), $CaCl_2$ (0.1%, w/w) or dextrose (5%, w/w) respectively in their drinking water, 2 weeks prior to sacrifice for experiments.

Castration within 24 hr after birth was performed under hypothermic conditions according to the procedure of Pfeiffer [13], whereas castration or adrenalectomy of adult rats was performed as previously de-

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scribed [14, 15]. Thyroparathyroidectomy was performed under ether anesthesia through the midline of anterior cervical location and hypophysectomy was performed via the auditory canal. The completeness of thyroparathyroidectomy was assessed by radioimmunoassay of plasma thyroxine concentration (T_4) (<0.6 μg%). Hypophysectomy was monitored by direct examination of the pituitary stalk under the median eminence at the time of sacrifice. No significant weight gain was noted in these rats during experiments following thyroparathyroidectomy, whereas significant weight loss was noted in rats subjected to hypophysectomy. Unless otherwise specified, the time period between surgical operation and the actual analysis of enzyme activity ranged from 4 to 5 weeks.

Isolation of hepatic microsomes and the determination of the apparent K_m *and the responsiveness of ethylmorphine* N*-demethylase.* Animals were killed by cervical dislocation. The fresh liver microsomes were prepared and used immediately as described previously [10]. Ethylmorphine *N*-demethylase activity derived from 400 mg liver tissue was determined in a final 2 ml incubation medium containing 1 μmole NADP, 40 μmoles glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 5 μmoles of $MgCl_2$, 10 μmoles of nicotinamide, 20 μmoles of semicarbazide, 90 μmoles of sodium phosphate and various concentrations of ethylmorphine-HCl (ranged from 0 to 4 mM). The apparent K_m and the responsiveness of ethylmorphine *N*-demethylase to androgen induction in adulthood were determined as previously described [10]. Incubation mixtures containing all of the assay ingredients except that of the microsomal enzymes were preincubated at 37° for 2 min. The reaction initiated by the addition of 0.2 ml microsomal enzyme suspension (3–5 mg protein) was allowed to proceed for 10 min. In order to minimize differences due to assay conditions, the kinetic analysis of this enzyme activity was performed with prior random arrangement of microsomes isolated from rats with various neonatal or pubertal pretreatment. Data obtained from each determination were computed for apparent K_m and V_{max} by a FORTRAN program as described by Cleveland [16]. Values were pooled from all experiments and subjected to statistical analysis and the Student *t*-test for the mean ± standard error and the degree of significance.

Determination of the turnover rates of the hepatic microsomal cytochrome P-450. The heme portion of cytochrome P-450 was labeled with 3,5-[^{3}H]-δ-aminolevulinic acid (δ-ALA) *in vivo* according to the method described by Levin *et al.* [11] with slight modification. Male and female rats of intact or hypophysectomized groups were used in these experiments. 3,5-[^{3}H]-δ-ALA (505 mCi/m-mole) was dissolved in 0.9% NaCl and administered to rats through the jugular vein under ether anesthesia, at a dose of 1.87 μmoles/kg. Animals were sacrificed at different times up to 120 hr. For the cytochrome P-450 turnover studies, hepatic 9000 *g* supernatants were prepared as described previously [10] and immediately frozen under liquid nitrogen and stored at −20°. This storage condition did not result in any significant changes of both of the ethylmorphine *N*-demethylase and aniline hydroxylase activities during 2-weeks storage period. Immediately before use, the 9000 *g* supernatant was thawed and centrifuged at 105,000 *g* for 1 hr to obtain the microsomal pellet. The microsomal pellet was washed once with 1.15% KCl–0.01 M sodium phosphate buffer (pH 7.4) and resuspended in the same buffer to give a final concentration of 200 mg liver per ml. This microsomal suspension was then incubated with 0.2% steapsin at 37° for 1 hr to solubilize cytochrome b_5 fraction [17]. The microsomal suspension was then resedimented at 160,000 *g* for 1 hr and the pellet was resuspended in 1.15% KCl–0.01 M sodium phosphate buffer. An aliquot of this microsomal suspension was then used for determining tritium radioactivity (Beckmann Scintillation Spectrophotometer LS-150) as previously described [14]. Another aliquot was used for the determination of protein concentration according to the Lowry's method with bovine serum albumin as standard [18] and cytochrome P-450 concentration according to Omura and Sato [19].

Chemicals and enzymes. Testosterone propionate (TP), estradiol dipropionate, δ-ALA, steapsin and NADPH-generating system (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) were purchased from Sigma Co. Ethylmorphine·HCl was provided by Merck & Co., Inc. Nicotinamide was obtained from Mann Research Laboratory. Semicarbazide·HCl and sodium hydrosulfite was obtained from Fisher Scientific Company. 3,5-[^{3}H]-δ-ALA (5.05 Ci/m-mole) was purchased from New England Nuclear and the purity of this label was routinely determined on thin layer chromatography in the solvent systems of *n*-butanol–acetic acid–H_2O (25:4:10) on a cellulose plate prior to use. The purity of 3,5-[^{3}H]-δ-ALA is >98 per cent.

RESULTS

Two sex-dependent characteristics, the apparent K_m of ethylmorphine *N*-demethylase and the responsiveness of this enzyme to androgen stimulation (abbreviated as K_m and V_{max}, respectively) in adult rat liver microsomes, have been reported previously by our laboratory as imprinted by neonatal androgen exposure [10]. Utilizing these two defined parameters as the end-points, we found that the critical period for imprinting K_m in the adult male rat is at days 2 and 4 of age, whereas the V_{max} is imprinted before days 12 and 14 of age (Table 1). Estradiol-17β dipropionate (days 2 and 4), an effective agent for the central nervous system imprinting [12], failed to imprint the male characteristics of hepatic monooxygenase system with respect to both K_m and V_{max}.

The maintenance of the imprinted K_m values in intact adult male rats was also studied. With the exception of hypophysectomy, male rats castrated, adrenalectomized, or thyroparathyroidectomized in adulthood maintain an imprinted male character of the K_m. Hypophysectomy of the male in adulthood, however, results in a shift of the K_m from male toward that of the female value. Females hypophysectomized as adults did not change appreciably the K_m of this enzyme as compared to the non-operated controls (Table 2). Additional experiments indicate that differences between neonatal androgen-treated (imprinted) and non-androgen-treated (non-imprinted) rats with

Table 1. The timing and steroid specificity for the programming of the basal apparent Michaelis constant (K_m) and the androgen-induced responsiveness of ethylmorphine *N*-demethylase activity in adult rats

Condition‡	Adult injection§	Ethylmorphine *N*-demethylase† Apparent K_m (mM)	V_{max} (nmoles HCHO/mg protein/10 min)
Male rats castrated at birth			
+ Corn oil (days 2 and 4)	Oil	0.74 ± 0.10*	20.4 ± 1.5*
	TP	0.43 ± 0.13	40.3 ± 6.8
+ TP (days 2 and 4)	Oil	0.31 ± 0.04**	28.2 ± 2.4**
	TP	0.41 ± 0.03	69.1 ± 8.1
+ TP (days 12 and 14)	Oil	1.19 ± 0.24	23.9 ± 1.7
	TP	0.50 ± 0.04	76.7 ± 7.3
+ TP (days 22 and 24)	Oil	1.36	23.7
	TP	0.65	43.6
+ EP (days 2 and 4)	Oil	1.62 ± 0.31	20.6 ± 0.5
	TP	0.74 ± 0.18	38.5 ± 3.9
Intact adult male rats	Oil	0.35 ± 0.15	79.5 ± 13.4

† Values represent the average or average ± S.E. of 2 or 4 determinations with exceptions of * is from 10 determinations and ** is from 7 determinations.

‡ TP: testosterone propionate, 500 μg/day for days 2, 4, 12 and 14 or 5000 μg/day for days 22 and 24. EP: 17β-estradiol dipropionate (20 μg/day) for days 2 and 4. Corn oil: 50 μl/day for days 2 and 4.

§ In adulthood, either daily corn oil (0.2 ml) or TP (10 mg) was given to these rats for a period of 10 days.

respect to K_m and V_{max} were also abolished by hypophysectomy (Table 3).

Levin *et al.* [11] have recently reported sex-dependent and neonatal androgen-imprinted differences of the turnover of hepatic cytochrome P-450 in rats. We have confirmed their results on the differences of turnover of hepatic cytochrome P-450 between male and female rats (Fig. 1A). A biphasic turnover of hepatic cytochrome P-450 with a fast phase *t*-1/2 of 6 hr and a slow phase *t*-1/2 of 66–70 hr was found in male and female rats. To learn if the sex-dependent differences in the turnover of hepatic cytochrome P-450 are also under the control of the hypothalamic–pituitary axis, male or female rats were hypophysectomized as adult and the turnover of cytochrome P-450 was studied (Fig. 1B). No appreciable changes were found in both of the slow and fast *t*-1/2's of the turnover of cytochrome P-450 between hypophysectomized and intact control rats. A summary of the effects of hypophysectomy on the turnover of hepatic cytochrome P-450 is presented in Table 4. Hypophysectomy markedly reduced the body weights and liver weights of these animals. The total microsomal protein content, however, was not affected by this surgery. Intact control male and female rats differed in their cytochrome P-450 turnover, with a value of fast/

Table 2. Effects of castration, adrenalectomy, thyroparathyroidectomy or hypophysectomy on the basal apparent K_m of hepatic microsomal ethylmorphine *N*-demethylase

Condition*	Ethylmorphine *N*-demethylase Apparent K_m (mM)
Adult Male	
Sham-operated (6)	0.37 ± 0.05
Castrated (2)	0.35
Adrenalectomized (5)	0.46 ± 0.02
Thyroparathyroidectomized (5)	0.36 ± 0.04
Hypophysectomized (4)	0.98 ± 0.05†
Adult Female	
Non-operated (3)	0.95 ± 0.13†
Hypophysectomized (3)	0.80 ± 0.10†

* Number in parentheses represents the total number of animals per group. All rats were operated in adulthood (60 days) and assessed 1 month later.

† Statistically significant at $P < 0.01$ as compared to sham-operated adult male rats.

Table 3. Effects of hypophysectomy in adulthood on the neonatal androgen-induced imprinting of the apparent K_m and responsiveness of hepatic microsomal ethylmorphine *N*-demethylase activity

Condition* Male rats castrated at birth	Adult Injection†	Ethylmorphine *N*-demethylase K_m (mM)	V_{max} (nmoles HCHO/mg prot./10 min)
Control			
Neonatal non-androgen treated	Oil (8)	0.88 ± 0.15	21.1 ± 5.1
	TP (4)	0.43 ± 0.10	29.9 ± 6.0
Neonatal androgen-tjeated	Oil (5)	0.34 ± 0.06‡	33.1 ± 5.0
	TP (5)	0.41 ± 0.03	50.7 ± 5.2§
Hypophysectomized			
Neonatal non-androgen treated	Oil (6)	1.15 ± 0.24	31.9 ± 5.4
	TP (6)	1.19 ± 0.24	28.9 ± 4.5
Neonatal androgen-treated	Oil (6)	0.95 ± 0.14	21.6 ± 2.6
	TP (6)	0.99 ± 0.11	33.5 ± 2.8

* Rats were castrated at birth and treated neonatally with either androgen (TP) or corn oil at days 2 and 4 (see Methods). Hypophysectomy was performed in adulthood.

† At adulthood, rats were treated daily with corn oil (0.2 ml) or TP (2 mg) for 10 days. Enzyme activity was measured as described in the Methods.

‡ Significantly different from the basal apparent K_m of neonatal non-androgen treated controls at $P < 0.05$.

§ Significantly different from the responsiveness of the neonatal non-androgen treated controls at $P < 0.1$.

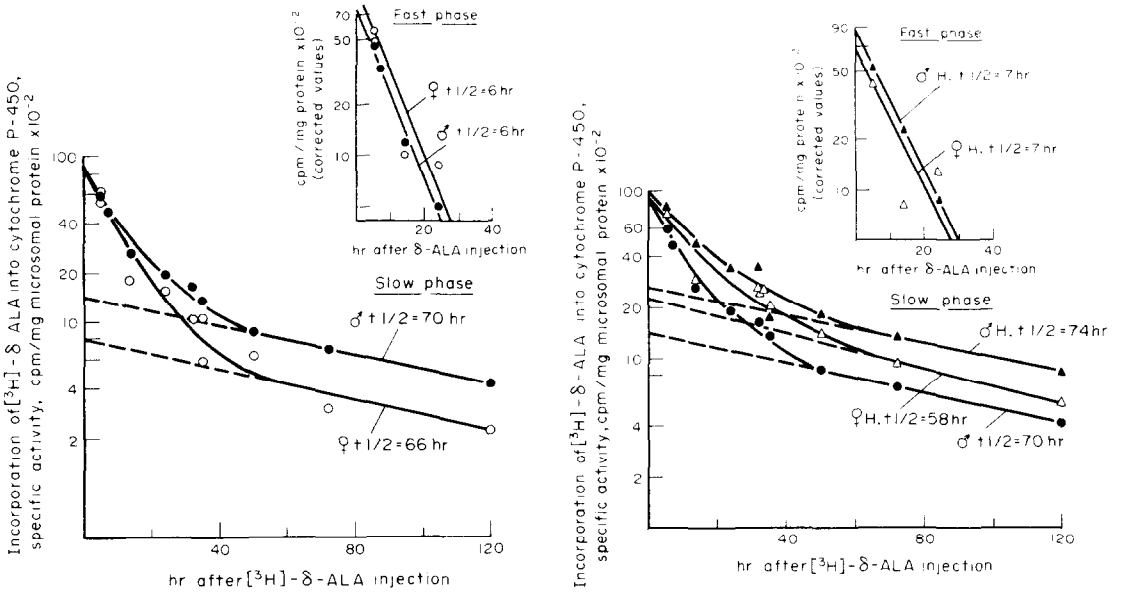

Fig. 1. (A) The turnover of cytochrome P-450 in hepatic microsomes of normal male and female rats: normal male: ♂ ●——●; normal female: ♀ ○——○. (B) The turnover of cytochrome P-450 in hepatic microsomes of normal male and hypophysectomized male and female rats: normal male: ♂ ●——●; hypophysectomized male: ♂H ▲——▲; hypophysectomized female: ♀H △——△; Each point appearing in Fig. 1 (A) and (B) represents the value obtained from one animal.

slow ratio at 5.8 and 11.3, respectively. Hypophysectomy increases the proportion of the slow turnover form of cytochrome P-450 resulting in a decreased fast/slow ratio to 2.7 and 2.9 in the male and female rats, respectively. This increases the proportion of the slow turnover form of cytochrome P-450 in hepatic microsomes of hypophysectomized rats and is accompanied by a significant increase in the content of hepatic cytochrome P-450 per mg of microsomal protein.

DISCUSSION

Several aspects of the neonatal imprinting of hepatic microsomal enzyme systems to metabolize certain xenobiotics are characterized. Since the sensitive period for imprinting of the apparent K_m differs from that of the V_{max}, it is suggested that these two imprinted parameters are independent. The differences in the responsiveness to androgen treatment in adulthood between neonatal androgen-treated and non-androgen-treated rats with respect to their hepatic microsomal ethylmorphine *N*-demethylase activity may represent a difference in net enzyme concentration since the apparent K_m of this enzyme becomes indistinguishable in these rats following repetitive androgen induction in adulthood.

Studies on the steroid specificity of neonatal imprinting on the hepatic microsomal monooxygenase system reveal that the imprinting in the central nervous system differs from that of the liver. Neonatal exposure to estradiol dipropionate (20 μg/rat) failed to imprint the hepatic ethylmorphine *N*-demethylase with respect to both of its apparent K_m and responsiveness. Although the dose of estradiol dipropionate used in this study is many-fold less than that of the testosterone propionate, this dose of estrogen is near the toxic level (unpublished observation) and has been demonstrated as effective in masculinizing the female brain [12]. This difference with steroid specificity between neonatal imprinting of the brain and that of several hepatic steroid-metabolizing enzyme activities has recently been reported by Gustafsson and Stenberg [20].

Table 4. Effects of hypophysectomy on the turnover of hepatic microsomal cytochrome P-450

Condition* (g.b. wt.)	Liver wt	Microsomal protein content (mg/g liver)	Cytochrome P-450 content (nmoles/mg prot)	Turnover of cytochrome P-450† (Fast/Slow Ratio)	T-1/2 Fast phase (hr)	T-1/2 Slow phase (hr)
Male, control (9) (290 ± 1)	12.3 ± 0.6	20.5 ± 2.2	0.49 ± 0.04	5.8	6	70
Male, hypophysectomized (7) (126 ± 2)	4.3 ± 0.2	19.1 ± 2.7	0.78 ± 0.12	2.7	7	74
Female control (10) (182 ± 2)	8.2 ± 0.4	20.7 ± 1.2	0.41 ± 0.05	11.3	6	66
Female, hypophysectomized (10) (117 ± 2)	3.9 ± 0.1	20.0 ± 0.5	0.69 ± 0.04	2.9	7	58

* Both male and female rats were hypophysectomized at age 40 days. The experiments were done 1.5 months after surgery. Numbers appearing in parentheses represent the total number of rats in each group.

† Turnover of hepatic cytochrome P-450 was determined by the method described by Levin *et al.* [11] with slight modification (see Methods).

Maintenance of these imprinted male characteristics (K_m, V_{max} and the turnover of hepatic cytochrome P-450) requires the presence of a pituitary gland. The fact that castration, adrenalectomy or thyroparathyroidectomy in adulthood did not affect the K_m in male rats; and treatment in adulthood with androgen to the neonatal non-androgen-treated rats [10], or with estrogen in intact male rats [21] decreased or increased respectively the K_m's, while both sex steroids are known to depress plasma concentrations of FSH and LH. These results preclude the mediator roles of ACTH, TSH, LH and FSH. Kramer *et al.* [22] have also reported that the presence of intact pituitary glands in adult male and female rats is essential for the androgen-induced responsiveness of ethylmorphine metabolism by hepatic microsomes. Gustafsson *et al.* [23] have recently obtained evidence that a factor termed feminotropin which is derived from pituitary extract, is distinctively different from FSH, LH, TSH, growth hormone or prolactin, but possesses feminizing activity on the steroid-metabolizing enzyme activities in cultured hepatoma cells.

The changes induced in the apparent K_m of ethylmorphine *N*-demethylase by sex steroids in adult male rats, can be interpreted as secondary to the release of certain pituitary factor(s), because similar androgen or estrogen injections failed to modify the apparent K_m of this enzyme in normal male rats hypophysectomized as adult (apparent K_m's are 0.85 and 0.95, respectively). Moreover, the neonatal androgen-imprinted differences in the pituitary factor(s) may be of a quantitative rather than qualitative nature, since neonatal non-androgen-treated male rats are capable of responding to androgen treatment by lowering their apparent K_m's of ethylmorphine *N*-demethylase to that of the male value.

Levin *et al.* [11] have recently reported a sex-dependent difference in the turnover (ratios of fast/slow) of hepatic microsomal cytochrome P-450 and attributed this difference to neonatal imprinting. In a series of publications, Gustafsson and Stenberg [7, 23–25] have described several types of hepatic microsomal steroid hydroxylase and reductase activities in adult male rats that are irreversibly programmed by gonadal hormones. They have further postulated the existence of pituitary feminizing factor(s) [26] and masculinizing factor(s) [23] which may mediate the action of exogenous or endogenous gonadal hormones on the hepatic microsomal steroid-metabolizing enzyme system. To analyze the relationship between the programming of the kinetic properties of hepatic microsomal ethylmorphine *N*-demethylase and that of the turnover of hepatic cytochrome P-450, we have studied the effects of hypophysectomy on the turnover of hepatic microsomal cytochrome P-450 in male and female rats. We have confirmed the finding of Levin *et al.* [11] that adult male and female rats differed in their hepatic cytochrome P-450 turnover with a fast/slow ratio of 5.8 and 11.3, respectively. Our results differ somewhat on the absolute values of fast/slow ratio of cytochrome P-450 turnover as that described by Levin and coworkers; this may result from differences of experimental conditions. Hypophysectomy, however, abolished the sex-dependent differences in the turnover of hepatic cytochrome P-450. Although the maintenance of this imprinted male character also requires the presence of an intact pituitary gland, the biochemical properties of the pituitary factor(s) may be of different nature. The K_m and V_{max} of ethylmorphine *N*-demethylase being maintained by masculinizing factor(s) whereas the turnover of hepatic microsomal cytochrome P-450 is being supported by feminizing factor(s). This difference need not preclude the relationship between these two imprinted parameters since the methodology employed in the present study is not sufficiently sensitive to delineate the neonatal androgen-imprinted differences of male characteristics at a molecular level. The possible relationship between these neonatal androgen-imprinted biochemical characteristics and the presence of certain sex specific form(s) of cytochrome P-450 is presently under investigation.

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