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CHANGES IN INDICES OF RAT TESTICULAR HEME BIOSYNTHESIS AND MICROSOMAL CYTOCHROME P-450 DURING SEXUAL MATURATION*

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Abstract—The levels of microsomal cytochrome P-450 and heme were measured in the rats testes from weaning (22 days) through attainment of sexual maturity (60–70 days) and beyond (70–125 days). Microsomal heme and cytochrome P-450 increased from 22 days of age to attain maximal levels at 40 days, the approximate age when rats enter the pubertal stage of sexual development. Microsomal heme and cytochrome P-450 levels then declined to attain plateau levels at approximately 65–80 days of age. Testicular mitochondrial δ-aminolaevulinate (ALA) synthase (EC 2.3.1.37) activity was shown to decrease from 22 days and to attain a plateau level at approximately 65 days of age. Administration of human chorionic gonadotropin (HCG) increased testicular ALA synthase activity at 49 and 89 days of age, but it failed to alter activity of this enzyme in prepubertal rats of 24 and 37 days of age. Testicular uroporphyrinogen (uro) I synthase (EC 4.3.1.8) activity was also shown to diminish from weaning through sexual maturity. The activity of uro I synthase was not affected by treatment with HCG at any age tested. These data suggest that testicular heme synthesis undergoes a change in regulation during sexual maturation.

Cytochrome P-450, a hemoprotein, functions as the terminal oxidase for various hydroxylation reactions of testicular androgen biosynthesis [1, 2]. The levels of rat testicular mitochondrial and microsomal cytochrome P-450 and the activities of the P-450-mediated enzymatic reactions increase after administration of human chorionic gonadotropin (HCG) and decrease after hypophysectomy [2–4]. It has been demonstrated previously in this laboratory that the activity of rat testicular mitochondrial δ-aminolaevulinate (ALA) synthase (EC 2.3.1.37) increases 24 hr after treatment with HCG [5], preceding the reported increases of cytochrome P-450 [2, 3]. These findings suggest that gonadotropin-mediated increases of cytochrome P-450 result from increased synthesis of testicular heme. It is possible, therefore, that alterations of testicular heme synthesis play an important role in the regulation of androgen synthesis. Regulation of androgen biosynthesis in the rat is known to undergo a major change during sexual maturation [6]. Plasma androgen levels increase rapidly from 20 days of age to attain maximal levels at approximately 40 days [6]. The mechanism responsible for this increase in androgen synthesis has not been fully elucidated. Because testicular cytochrome P-450 is intimately involved in the biosynthesis of androgens, we have examined the levels of this cytochrome and the various indices involved with the regulation of rat testicular heme synthesis at various ages.

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MATERIALS AND METHODS

Materials. Pyridoxal-5′-phosphate, disodium EDTA, sodium succinate, porcine heart succinyl-CoA synthetase (EC 6.2.1.4) and human chorionic gonadotropin (HCG) were obtained from the Sigma Chemical Co., St. Louis, MO. GTP, δ-aminolevulinic acid (ALA) and coenzyme A were obtained from CalBiochem, La Jolla, CA. [2,3-^{14}C]Succinic acid (19 mCi/mmole) was obtained from ICN Pharmaceuticals, Irvine, CA. Other chemicals were reagent grade.

Treatment of animals. Male Sprague–Dawley rats of specified ages were obtained from Sasco, Inc., Omaha, NE. Animals were permitted food and water *ad lib.* HCG was dissolved in 0.9% NaCl and administered subcutaneously (100 units; 2.5 ml/kg) at 12-hr intervals. Control animals received equal volumes of 0.9% NaCl at 12-hr intervals. Animals were killed during the same time of day (8:00 to 9:00 a.m.) to avoid any difference in effects caused by circadian rhythms.

Preparation of tissues. Animals were decapitated and the testes were removed, decapsulated, and weighed. The testes were pooled from four rats for ages less than 30 days, two rats for ages 31–50 days, and one rat for ages exceeding 50 days. For animals less than 50 days of age, values represent the mean for a minimum of three different sample pools to enable calculation of a true S.E.M. Testicular homogenates (10%, w/v) were prepared in 0.25 M sucrose. Subcellular fractions were prepared by the method

of Cammer and Estabrook [7]. Spectrophotometric assay indicated that microsomal preparations were devoid of hemoglobin. Microsomal preparations were devoid of the mitochondrial marker enzyme, glutamate dehydrogenase [5]. Protein was determined by the method of Bradford [8], using bovine serum albumin as the standard.

Microsomal heme determination. Microsomal heme was determined from the difference spectrum of the oxidized/reduced pyridine hemochromogen between 541 and 557 nm, using a millimolar extinction coefficient of 20.7 mM^{-1} cm^{-1} [9].

Microsomal cytochrome P-450 determination. Levels of cytochrome P-450 were determined from the carbon monoxide difference spectrum (450–490 nm) of dithionite-reduced microsomes, using an extinction coefficient of 91 mM^{-1} cm^{-1} [10].

ALA synthase activity. Testicular mitochondrial ALA synthase activity was determined by a radioisotopic method employing [2,3-^{14}C]succinate and a succinyl-CoA generating system developed previously in this laboratory [5]. Recovery of ALA was 91 per cent, and the identity of the ^{14}C-labeled material was confirmed as the [^{14}C]ALA-pyrrole by thin-layer chromatography in two different solvent systems and authentic ALA-pyrrole standard [5]. Rat testicular ALA synthase activity was found to be localized predominantly in the mitochondrial fraction [5].

Uroporphyrinogen synthetase activity. Testicular uroporphyrinogen (uro) I synthase (EC 4.3.1.8) activity was determined in the cytosolic fraction by the method of Strand *et al.* [11]. Product formation was found to be linear for 80 min with up to 0.5 mg of cytosolic protein.

Statistics. Data were analyzed by Student's *t*-test to determine the significance of difference between means.

RESULTS

The levels of testicular microsomal cytochrome P-450 in rats from 22 days of age through sexual maturity are depicted in Fig. 1. Microsomal cytochrome P-450 attained a maximum value at 40 days followed by a rapid decline to a plateau beginning at approximately 65–80 days. This developmental pattern is similar to that for hepatic microsomal cytochrome P-450 [12]. Levels of microsomal cytochrome P-450 have also been examined in the ovary and adrenal of the female rat [13]; the levels of microsomal cytochrome P-450 were found to undergo a more gradual elevation from approximately 10–20 days to attain a plateau at approximately 40–60 days of age [13].

Testicular microsomal heme exhibited the same developmental pattern during sexual maturation as did cytochrome P-450 (Fig. 2). The levels of microsomal heme also reached a peak at 40 days, followed by a decline to a plateau at approximately 65–80 days. These results suggest that the increase of cytochrome P-450 during sexual maturation may be related to an increased synthesis of testicular heme.

Because increases in the levels of heme and cytochrome P-450 may be associated with an increase of ALA synthase activity [14], the activity of this enzyme was measured at various ages. As shown in Fig. 3, the activity of testicular ALA synthase declined from 22 days and attained a plateau at approximately 60 days of age.

It was demonstrated previously that the administration of HCG to mature rats results in an increase in testicular ALA synthase activity [5]. HCG was administered for 2 days to rats of various ages to determine the effects of this gonadotropin on testicular ALA synthase activity during sexual maturation. Table 1 illustrates that HCG had no effect on testicular ALA synthase activity in rats of 24 and 37 days of age. In rats of 49 and 89 days of age, however, ALA synthase activity increased 97 and 73 per cent respectively. These data suggest that the regulation of testicular ALA synthase activity by gonadotropins differs with age. This apparent change in the regulation of testicular ALA synthase activity appears to occur between 37 and 49 days of age, a period when the levels of microsomal heme and cytochrome P-450 are switching from a rapid increase to a decline.

The elevated levels of ALA synthase activity in the immature rat suggested that perhaps another

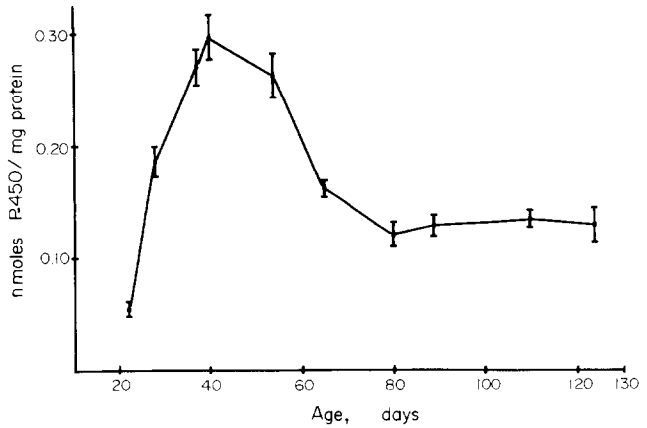

Fig. 1. Levels of rat testicular microsomal cytochrome P-450 as a function of age. Animals were killed at the specified age, and microsomal cytochrome P-450 was determined as described in Materials and Methods. Each point is the mean ± S.E.M. of four to six determinations.

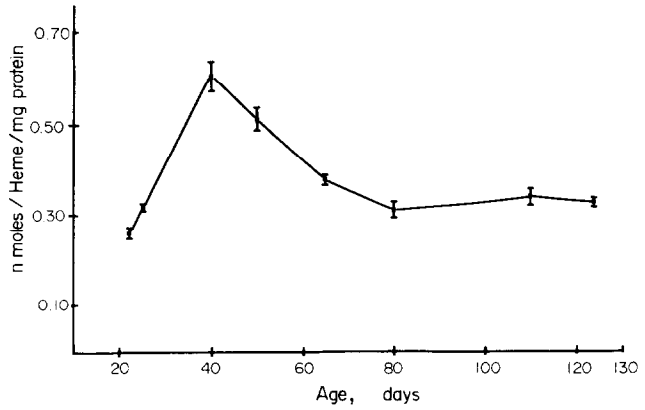

Fig. 2. Levels of rat testicular microsomal heme as a function of age. Animals were killed at the specified age, and microsomal heme was measured as described in Materials and Methods. Each point is the mean ± S.E.M. of three to four determinations.

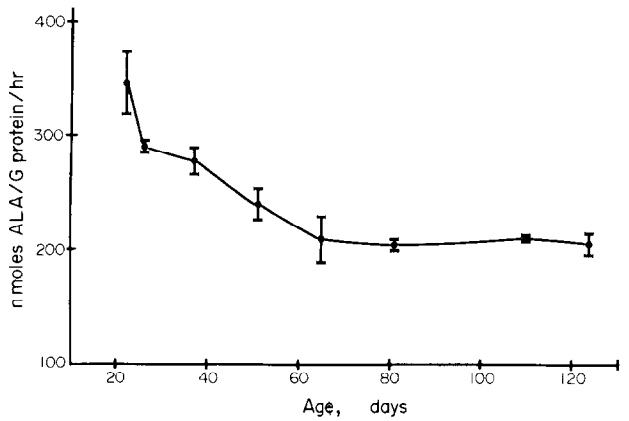

Fig. 3. Activity of rat testicular mitochondrial ALA synthase as a function of age. Animals were killed at the specified age, and ALA synthase activity was measured as described in Materials and Methods. Each point is the mean ± S.E.M. of three to four determinations.

Table 1. Effect of HCG on rat testicular ALA synthase activity in rats of various ages*

Age (days)	ALA synthase activity [nmoles ALA·(g protein)$^{-1}$·hr^{-1}]	
	Control	HCG
24	315 ± 30	293 ± 20
37	292 ± 13	318 ± 11
49	230 ± 18	453 ± 49†
89	214 ± 12	370 ± 23†

* Rats of appropriate ages received HCG (100 units, s.c.) at 12-hr intervals for 2 days prior to being killed. The ages listed are the ages at sacrifice. Testicular mitochondrial ALA synthase activity was determined as described in Materials and Methods. Each value is the mean ± S.E.M. of three determinations.

† Denotes a significant difference ($P < 0.05$) between HCG-treated and control rats.

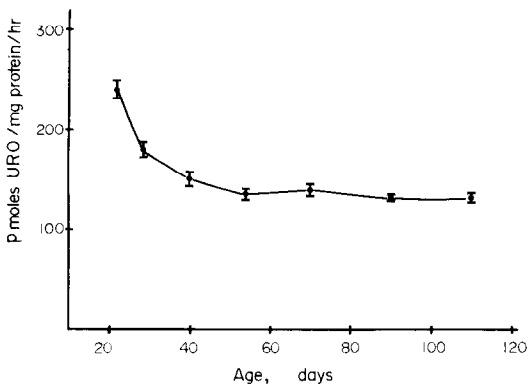

Fig. 4. Activity of rat testicular uroporphyrinogen I synthase as a function of age. Animals were killed at the specified age, and uroporphyrinogen I synthase activity was measured as described in Materials and Methods. Each point is the mean ± S.E.M. of three determinations.

Table 2. Effect of HCG on rat testicular uroporphyrinogen I synthase activity in rats of various ages*

Age (days)	Uroporphyrinogen I synthase activity [pmoles uro·(mg protein)$^{-1}$·hr^{-1}]	
	Control	HCG
24	215 ± 9	212 ± 6
37	174 ± 4	183 ± 8
49	154 ± 1	166 ± 3
89	123 ± 1	124 ± 2

* Rats of appropriate ages received HCG (100 units, s.c.) at 12-hr intervals for 2 days prior to being killed. The ages listed are the ages at sacrifice. Testicular uroporphyrinogen I synthase activity was determined as described in Materials and Methods. Each value is the mean ± S.E.M. of three determinations.

enzyme in the testicular biosynthetic pathway might be rate-limiting for the synthesis of heme. Uro I synthase has been shown to be present in liver at a very low activity and under certain circumstances may be rate limiting for hepatic heme synthesis [14]. For this reason, the activity of this enzyme was measured in the developing rat testis (Fig. 4). The activity of testicular uro I synthase, like that of ALA synthase, was found to be higher in the weanling than in the adult. Testicular uro I synthase activity declined from 22 days and reached a plateau at approximately 60 days. Thus, the relative activities of these two enzymes appear to remain constant. Therefore, it seems unlikely that uro I synthase acts as the rate-limiting enzyme in heme synthesis in the immature rat testes.

The potential inducibility of testicular uroi I synthase by HCG was investigated at various ages (Table 2). The activity of this enzyme was not altered by HCG at any of the ages examined.

DISCUSSION

The activity of hepatic ALA synthase has been reported to be higher in the fetal rat than in the adult and to decline throughout the perinatal period to reach adult levels by 3–4 days postpartum [15]. It has also been reported that both fetal and perinatal rat hepatic ALA synthase are not susceptible to induction by compounds known to increase adult hepatic ALA synthase activity [16]. It has been shown herein that testicular ALA synthase activity was higher in the weanling rat, declining to much lower values in the sexually mature animal. Furthermore, ALA synthase activity in the immature rat was not increased by administration of HCG as it was in the mature rat. Thus, the alterations observed in the activity and regulation of testicular ALA synthase during sexual maturation appeared to be analogous to those of hepatic ALA synthase during the perinatal period.

The decline in testicular ALA synthase activity at a time when microsomal heme and cytochrome P-450 were increasing and the lack of an effect of HCG on this enzyme in the immature rat suggest that ALA synthase may not be rate limiting for the synthesis of heme in the immature rat testis.

It is known that testicular steroidogenesis undergoes significant changes during sexual maturation [6, 17]. Between the ages of 25 and 45 days, the major product of the rat testis switches from 5α-reduced androgens to testosterone [18]. The increases of testicular microsomal heme and cytochrome P-450 during sexual development reported herein coincide with the rise in plasma testosterone levels. Thus, it is possible that an alteration of testicular heme synthesis during sexual maturation, resulting in an increase in cytochrome P-450, may be involved in the increase of testosterone synthesis.

The age period when rapid changes in microsomal heme and cytochrome P-450 occur, 40–65 days, is a period studied by many investigators concerned with various aspects of testicular function. Variation in the levels of cytochrome P-450, known to mediate various steroid hydroxylations, may be of importance in the evaluation and the interpretation of research findings obtained during this period of sexual development.

The mechanism responsible for the rapid increase in testicular microsomal heme and cytochrome P-450 during sexual maturation is not known. Future work in this laboratory will be concerned with investigations into regulation of the testicular heme pathway during sexual maturation.

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