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DEHYDROEPIANDROSTERONE (DHA) INDUCED PRECOCIOUS OVULATION: CORRELATIVE CHANGES IN BLOOD STEROIDS, GONADOTROPINS AND CYTOSOL ESTRADIOL RECEPTORS OF ANTERIOR PITUITARY GLAND AND HYPOTHALAMUS*

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SUMMARY

The administration of dehydroepiandrosterone (DHA) to immature female rats results in a precocious ovulation followed by ovulatory failure. Earlier studies suggested that the peripheral conversion of DHA to estrogens may be involved in the induction of precocious ovulation. This study demonstrates a significant elevation in serum levels of DHA, androstenedione, testosterone, 5α-dihydrotestosterone (DHT), and estradiol within 2 h of DHA administration. That these changes were due to conversion of administered DHA and not due to secretion of endogenous steroids was suggested by no initial changes in the C-21 precursors such as 17 hydroxyprogesterone and progesterone. Furthermore, serum estradiol levels dropped dramatically after DHA withdrawal. The series of events leading to precocious ovulation, namely the rise in blood estradiol, the depletion of cytoplasmic estradiol receptors of the anterior pituitary gland and the hypothalamus, followed by the preovulatory surge of gonadotropins leading to ovulation, are similar to those found in pregnant mare serum gonadotropin (PMSG) induced precocious ovulation, the onset of natural puberty, and during ovulation in the adult cycling rat.

INTRODUCTION

A relationship between excessive androgen secretion and ovulatory failure has been suggested in the human in a number of clinical conditions such as polycystic ovarian disease [1] and virilizing adrenal and ovarian tumors [2–4]. In order to study the effects of androgens on ovulatory failure, an experimental model has been designed in our laboratory in which the administration of dehydroepiandrosterone to female rats caused ovulatory failure and formation of polycystic ovaries: termination of DHA treatment led to restoration of normal cyclicity and fertility after a lag period of several days [5–7]. DHA administration to immature rats led to a precocious ovulation between days 30-33 of life prior to the onset of ovulatory failure [8]. Our prior studies suggested that the conversion of DHA to estrogens was the likely mechanism for the induction of precocious puberty in the immature rat. This indirect conclusion was based on the fact that non-aromatizable androgens such as dihydrotestosterone (DHT) could not induce precocious ovulation and the administration of cyanoketone, (2α-cyano, 17β-hydroxy-4,4,17α-trimethyl-5-androsten-3-one) an inhibitor of 3β-hydroxysteroid dehydrogenase, was capable of blocking the precocious puberty induced by DHA treatment [8]. While prior studies of the DHA treated rat have dealt primarily with pituitary and serum gonadotropins there have been no studies concerning the temporal changes in blood steroids in relation to the induction of precocious puberty. Quantified in the current investigation were peripheral serum concentrations of several steroid hormones (estradiol, progesterone, 17 hydroxyprogesterone, DHA, testosterone, androstenedione and DHT), serum gonadotropins, and cytosol estradiol receptor levels of the anterior pituitary gland and hypothalamus during the experimental induction of precocious puberty. These parameters were elevated in order to afford a more complete understanding of the mechanisms whereby DHA induces the early onset of puberty in female rats.

MATERIALS AND METHODS

Female rats were received from the Holtzman Co. (Madison, Wisc.) at 23 days of age and were housed 3–4 per cage. Rats were given free access to lab chow and water. The lighting schedule allowed for 14 h light; 10 h darkness. Rats were injected subcutaneously with either DHA 60 mg/kg BW/day in pro-

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pylene glycol vehicle, or vehicle alone at 9 am on 27–29 days of age. The purity of DHA was verified prior to use and 6–7 rats were used for control and treatment groups. The various groups were sacrificed by ether inhalation at 11 am from 27–30 days of age. The ovaries and uteri were dissected, blotted and weighed to the nearest 0.5 mg. Oviducts were pressed between glass slides and examined microscopically in order to count ova. Blood was drawn by cardiac puncture and the serum obtained was stored at −15°C until the time of steroid and gonadotropin assay.

Hormone assays. Serum steroids were quantified by multiple steroid radioimmunoassay [9]. The recovery of serum steroids following extraction and celite column chromatography ranged from 65% (estradiol) to 91% (17 hydroxyprogesterone). Interassay variation (coefficient of variation) of this method ranged from 4.3% (androstenedione to 12.8% (DHA). FSH and LH were quantified by radioimmunoassay methods previously described [10] using materials supplied by the National Institute of Arthritis, Metabolic and Digestive Diseases, Hormone Distribution Program, National Institutes of Health, U.S. Public Health Service.

Cytosol estradiol receptor assays. Anterior pituitary glands and hypothalami were rapidly excised and placed directly into ice cold Tris–EDTA buffer (TE), (0.01 M Tris, 1.5 mM Na_2 EDTA, pH 8.0). Within 30 min of dissection, these tissues were homogenized in cold TE buffer (6 glands/1.8 ml; yielding a protein concentration of 1–2 mg/ml) using glass homogenizers (Kontes). Homogenates were centrifuged at 105,000 *g* (1 h, 4°C) in a Beckman L2 65B ultracentrifuge. Aliquots ($n = 4$ per treatment group) of the supernatants (cytosol) were incubated with saturating levels of radiolabeled estradiol (2,4,6,7,[^{3}H]-estradiol) for the determination of estradiol receptor concentration [11, 12]. Cytosol protein was measured by the method of Lowry *et al.* [13] and estradiol receptor levels were expressed as specific bound moles [^{3}H]-estradiol/mg cytosol protein. These determinations measure the unoccupied binding sites. Due to the rapid translocation of the estrogen–receptor complex to the nucleus, occupied binding sites comprise less than 3% of the total binding sites as determined by charcoal stripping of the cytosol before determination of binding sites (Muldoon, T. G. Personal communications). The cytosol from the pituitary gland and the hypothalamus did not show specific or high affinity binding with testosterone, DHT or DHA as contrasted to estradiol [14–16].

Statistics. Differences in the various experimental parameters between treated and control groups were analyzed by an unpaired Students *t*-test [17] and were considered significant at $P < 0.01$.

RESULTS

Blood steroid levels. The changes in blood steroids were studied on days 27–30 of life. This time schedule was selected because in three separate trials, vaginal opening occurred most consistently on day 30 in rats treated with DHA (age 27–29) with 91% of rats displaying estrous vaginal smears. Ova and/or corpora lutea were first detected on day 30 ($n = 26/38$). Although a large number of rats ovulated by day 30, ova were recovered as late as day 32 in rats treated with DHA. Vaginal opening or evidence of ovulation in untreated rats did not occur until at least 35 days of age.

The alterations in peripheral serum steroid levels resulting from short term DHA administration are compared to levels in vehicle-treated rats in Tables 1 and 2. Serum levels of DHA, androstenedione, testosterone and DHT were significantly increased over control levels ($P < 0.01$) within two h after the first DHA injection at 27 days of age (Table 1). Serum concentrations of these androgens remained significantly elevated through 29 days of age in DHA-treated rats. On the AM of day 30, (24 h after the last DHA injection) serum levels of DHA, testosterone, androstenedione, and DHT declined significantly. Concentrations of each androgen was still sig-

Table 1. Effects of short-term DHA or vehicle injections on peripheral serum steroid levels in immature female rats†

Age at sacrifice	Steroid concentrations (ng/ml serum) mean ± S.E.							
	Testosterone		Dehydroepiandrosterone		Androstenedione		Dihydrotestosterone	
	DHA	Vehicle	DHA	Vehicle	DHA	Vehicle	DHA	Vehicle
27 am	4.01 ± *	0.06 ±	95.63 ± *	0.27 ±	4.14 ± *	0.59 ±	0.92 ± *	0.06 ±
	0.01	0.01	18.61	0.02	0.08	0.18	0.15	0.03
28 am	3.83 ± *	0.05	68.79 ± *	0.43 ±	3.81 ± *	0.15 ±	0.62 ± *	0.04 ±
	0.17	0.01	2.72	0.20	0.41	0.07	0.05	0.02
29 am	4.91 ± *	0.07 ±	98.11 ±	lost	3.87 ± *	0.27 ±	0.59 ± *	0.04 ±
	0.24	0.02 ±	5.75		0.14	0.07	0.06	0.02
30 am	1.42 ±	0.06 ±	26.17 ± *	0.16 ±	1.06 ± *	0.29 ±	0.16 ± *	0.04 ±
	0.62	0.01	5.16	0.16	0.08	0.07	0.02	0.01

† There were at least 6 animals per treatment group for each sacrifice time; treatments were either DHA 60 mg/kg BW or vehicle on days 27–29 of life.

* Indicates significant differences between DHA and vehicle-treated controls at $P < 0.01$.

Table 2. Effects of short-term DHA or vehicle treatment on peripheral serum levels of estradiol, progesterone and 17 hydroxyprogesterone in immature female rats†

Age at sacrifice	Steroid concentrations (ng/ml serum) mean ± S.E.					
	Estradiol		Progesterone		17 Hydroxyprogesterone	
	DHA	Vehicle	DHA	Vehicle	DHA	Vehicle
27 am	0.25±*	0.04±	2.45±	2.09±	0.80±	0.81±
	0.06	0.02	1.20	1.31	0.05	0.22
28 am	1.21±	lost	3.05±	1.00±	0.75±	0.84±
	0.05		0.95	0.66	0.11	0.28
29 am	0.22±*	0.03±	7.72±*	4.19±	1.53±*	0.94±
	0.02	0.01	0.77	1.63	0.16	0.15
30 am	0.11±*	0.02±	1.44±	5.18±	1.16±	1.43±
	0.02	0.01	0.78	1.23	0.41	0.29

* Indicates significant differences between DHA and vehicle treated controls at $P < 0.01$.
† See Table 1.

nificantly higher in those rats treated previously with DHA than in vehicle-treated rats.

Within two h after the first DHA treatment, serum estradiol concentrations (Table 2) were significantly increased over levels in vehicle-treated controls and remained elevated through day 29. On the am of day 30 (24 h of DHA withdrawal), serum estradiol levels declined by 50% as compared to levels on Day 29. Serum levels of estradiol in the vehicle-treated (propylene glycol) rats in this study were somewhat higher than levels in untreated rats noted in a previous study of natural puberty [18]. This discrepancy could be due to factors such as handling and stress made necessary by the daily injections in the vehicle-treated group. Such an explanation is supported by the fact that the uterine weight in these animals showed a significant increase by day 29 ($P < 0.01$; Fig. 1) as compared to day 31 in untreated animals reported earlier [18]. Consistent with the increased level of serum estradiol found within two h of DHA administration, a significant increase ($P < 0.01$) of uterine weight was also observed (Fig. 1). The uterine weight of DHA treated animals rose progressively on day 28 and day 29 with uterine ballooning occurring on day 29. The uterine weights of vehicle-treated rats were significantly lower than DHA treated rats at all points of time studied. Ovarian weights during this period were unchanged in both groups with the exception of the DHA-treated group on day 30. In this group a significant increase in ovarian weight was noted coincident with the initial ovulation and formation of corpus luteum.

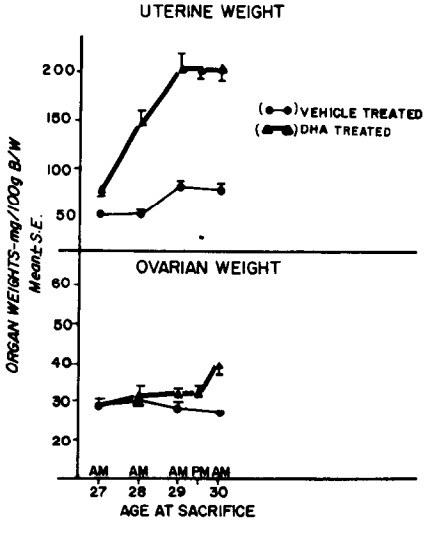

Fig. 1. Uterine and ovarian weights in female rats treated with DHA or vehicle starting on day 27 of age ($n = 6$–7 animals per group). Uterine weights were significantly elevated by day 28 of age ($P < 0.01$) while ovarian weights increased significantly on the morning of day 30 ($P < 0.01$).

Serum concentrations of progesterone and 17 hydroxyprogesterone were not significantly different in DHA-treated and vehicle-treated rats on days 27 and 28. There was however an increase in serum levels of both compounds in DHA-treated rats on day 29. On day 30, serum progesterone and 17 hydroxyprogesterone levels in DHA-treated rats had declined and were not significantly different from concentrations in vehicle-treated controls at this time. It would appear that the dramatic increase in levels of DHA, androstenedione, testosterone, DHT and estradiol within two h after the onset of DHA adminstration were the result of metabolism of the injected steroid, DHA. This contention is supported by the finding that no change in serum levels of C-21 precursors (17 hydroxyprogesterone and progesterone) were documented prior to day 29.

Cytosol estradiol receptors and serum gonadotropins. As shown in Fig. 2 (lower panel), DHA treatment beginning at 27 days of age caused a significant decrease ($P < 0.01$) in cytosol estradiol receptor levels of the anterior pituitary gland as compared to levels in vehicle-treated controls. Maximal receptor depletion occurred on the am of day 29. On day 30 (estrous), there was no significant replenishment of cytosol receptor binding capacity and serum estradiol levels were still significantly elevated. In a separate group of rats, hypothalamic cytosol estradiol receptor

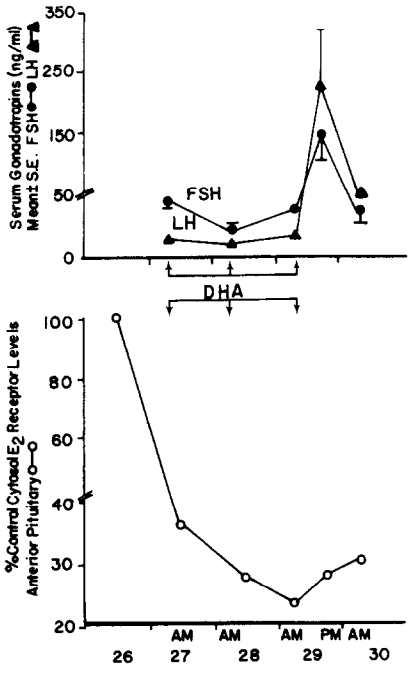

Fig. 2. Serum gonadotropins and anterior pituitary gland cytosol estradiol receptors were determined in the same animals (6 animals per time period). Control anterior pituitary gland receptor levels ranged from 7.1 ± 0.1 to 8.2 ± 0.1 × 1 × 10^{-14} mol [^{3}H]-E_2/mg cytosol protein. The standard errors in the values of anterior pituitary cytosol estradiol receptors were too small to graph.

levels were also found to be signficantly depleted ($p < 0.01$) after DHA administration (controls ranged from 8.1 ± 0.3 to 10.1 ± 0.1 × 10^{-15} mol/mg cytosol protein; DHA-treated ranged from 4.9 ± 0.2–5.8 ± 0.1 × 10^{-15} mol/mg cytosol protein). Serum levels of gonadotropins (Fig. 2, upper panel) were unaltered from day 27 through the am of day 29 during DHA administration. At 6 pm, day 29, serum levels of FSH and LH were significantly elevated in the DHA-treated rats as compared to concentrations at 11 am on day 29. On the morning of day 30, serum gonadotropins had declined.

DISCUSSION

The effects of androgenic steroids on reproductive processes of laboratory animals have been the subject of numerous investigations in the past. Many of these studies were undertaken primarily in order to ascertain the role of androgens in the infertile human female in whom ovulatory failure was associated with androgen excess.

Black and Mahesh[6] and Knudsen and Mahesh[7] have shown that short term treatment of prepubertal female rats with DHA advances the onset of puberty. The results of the current study confirm their findings in this regard. The administration of other aromatizable androgens such as androstenedione and testosterone [19] or estrogen [20] were also found to advance puberty in the female rat. There is ample evidence that DHA is converted to estrogens in the rat ovary [21–22]. Its conversion to estrogens in the monkey brain [23] and peripherally in the human has also been demonstrated [24, 25]. Longcope *et al.*[26] have demonstrated such a conversion of androgens in muscle and adipose tissue in the human.

In the current study, we have demonstrated that DHA administration to immature female rats causes a significant elevation in blood levels of estradiol (Table 2), in addition to increasing titers of androgen intermediates such as androstenedione and testosterone (Table 1). The increased levels of estradiol, as determined by radioimmunoassay, are further reflected in the rapid uterotropic effect following DHA adminstration (Fig. 1). It is suggested that the increased levels of estradiol were due to the metabolic conversion of DHA rather than via increased ovarian secretion. Previous studies have demonstrated that estrogen can arise through metabolism of exogenously administered DHA [21–26]. In addition, Knudsen and Mahesh[8] reported that the administration of cyanoketone, an inhibitor of 3β-hydroxysteroid dehydrogenase, was capable of blocking the precocious puberty induced by DHA treatment. In support of this hypothesis is the rapid decline in serum estradiol after DHA was discontinued (Table 2). Serum levels of progesterone and 17-hydroxyprogesterone, which are not metabolites of DHA and consequently could arise only through endogenous secretion, were unaffected initially by DHA treatment.

The mechanism whereby aromatizable androgens and estrogen may advance the onset of puberty are still not completely understood. The role of increased testosterone and androstenedione, other than to serve as intermediates for estrogen biosynthesis, is currently unknown. The preovulatory gonadotropin surge (Fig. 2) was preceded by a significant increase in serum estradiol (Table 2). Knudsen and Mahesh[8] also noted a significant increase in serum gonadotropins, at the time of DHA induced precocious ovulation. Similar relationships between serum estrogen and the gonadotropin surge has been noted in other animal preparations [27, 28].

The specific site(s) involved in steroid activation of goandotropin secretion are currently unresolved. Evidence has been presented suggesting steroid sensitive loci in both the hypothalamus and anterior pituitary gland [29, 30]. Since both tissues have been shown to possess estrogen concentrating mechanisms [15, 16], it is possible that hypothalamic and anterior pituitary estradiol receptors participate in the induction of puberty by DHA. In this study, we have demonstrated the temporal relationship between levels of cytosol estradiol receptors in the anterior pituitary gland and hypothalamus and the preovulatory FSH/LH surge. DHA treatment caused a significant reduction in cytosol estradiol receptor levels in both tissues, with maximal depletion occurring prior to the FSH/LH surge.

It has been shown that cytosol estradiol receptors of the uterus, hypothalamus and anterior pituitary gland are significantly lowered following exogenous estrogen administration to ovariectomized rats [11]. Similar significant depletion of anterior pituitary gland and hypothalamic estradiol receptors have been documented during increased endogenous estrogen secretions prior to the preovulatory gonadotropin surge in the PMSG-primed immature female rat [12], the untreated pubertal female rat [18], and the adult cycling rat [31]. The importance of cytosol binding accompanied by nuclear translocation of the estrogen–receptor complex in relation to the control of gonadotropin secretion is suggested by the fact that administration of an estrogen antagonist [32], or estradiol antibodies [32], both of which would be expected to antagonize the interaction of estradiol with its cytoplasmic receptor, are effective in blocking ovulation in the female rat.

It has recently been suggested that DHA may play an important role in triggering the onset of puberty in the human [34], but its mode of action in initiating these events is unclear. This study indicates that in the DHA-treated rat, the series of events leading to precocious ovulation and puberty, namely the rise in blood estradiol, the depletion of cytoplasmic estradiol receptors of the anterior pituitary gland and the hypothalamus followed by the preovulatory gonadotropin surge leading to ovulation are similar to those found in PMSG-primed precocious ovulation [12], the onset of natural puberty [18] and ovulation in the adult cycling rat [31].

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