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EFFECTS OF FETAL ALCOHOL EXPOSURE ON BRAIN 5α-REDUCTASE/AROMATASE ACTIVITY

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Summary—The local formation of the testosterone metabolites 5α-dihydrotestosterone and 17β-estradiol within the hypothalamic-preoptic area (HPOA) is essential for the normal sexual differentiation of the male central nervous system (CNS) during a perinatal critical period in the rat. Testosterone, the substrate for these reactions, is derived primarily from synthesis within the fetal testis. Fetal alcohol exposure (FAE) during this critical period profoundly affects fetal testicular steroidogenesis as well as the sexual differentiation of the CNS. The present study was conducted to determine whether FAE directly affects the local metabolism of androgens within the developing CNS or whether reduced androgen substrate, via a testicular lesion, is a more likely explanation for the known effects of FAE on the CNS. The enzymatic activities of 5α-reductase and aromatase were simultaneously quantitated in the newborn rat HPOA following FAE. Neither the enzymatic activity of 5α-reductase, aromatase nor their ratio were significantly influenced ($P > 0.05$) by FAE with respect to controls. FAE apparently does not alter the disposition of the androgens within the newborn rat HPOA. These results support the hypothesis that FAE alters the sexual differentiation of the CNS through inhibition of androgen biosynthesis at the level of the perinatal rat testis.

INTRODUCTION

Adult male rats exposed to ethanol *in utero* exhibit a reduction in the volume of the sexually dimorphic nucleus of the preoptic area [1–3], demasculinization and feminization of adult male sexual behavior patterns [2–5], and feminized patterns of maze learning [6], saccharin consumption [6, 7], and juvenile social play behavior [8]. The development of these sexually dimorphic behavior patterns is thought to be dependent upon the androgenic milieu of the fetus during the final week of gestation through the first week of postnatal life i.e. the critical period for sexual differentiation of the central nervous system (CNS). Testosterone biosynthesis in the fetal and neonatal testes is the primary hormonal influence acting during the first postnatal week and thus of key importance to the sexual differentiation of the male brain [1–8]. Inhibition of testicular steroidogenesis in the perinatal rat is one possible mechanism by which prenatal ethanol exposure might alter the androgen milieu of the perinatal rat and, therefore, the sexual differentiation of the CNS. Evidence supporting this hypothesis has been previously published [9, 10].

Another possible mechanism by which fetal alcohol exposure might alter the sexual differentiation of the CNS is through the altered metabolism of androgens within the brain itself. Within the hypothalamus, it appears that both androgens and estrogens are involved in brain sexual differentiation through the local conversion of circulating androgens either to more potent androgens (viz. the 5α-reductase pathway) or via the aromatase pathway to estrogens [11–16]. In order to investigate this latter hypothesis, we have developed a radiometric enzyme assay to simultaneously quantitate the activities of 5α-reductase and aromatase within the hypothalamic-preoptic area (HPOA) of newborn male rats. We have determined that *in utero* exposure to alcohol during Day 12 of gestation through parturition does not alter 5α-reductase activity, aromatase activity or their ratio.

EXPERIMENTAL

Chemicals

Steroid standards and nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH) were purchased from Sigma Chemical Company (St. Louis, Mo.) and their purity checked by high-performance liquid chromatography (HPLC). [1β-^{3}H]Androstene-3,17-dione (27.4 Ci/mmol), [1α, 2α-^{3}H]androstene-3,17-dione (44 Ci/mmol) and [1,2,6,7,16,17-^{3}H]testosterone (165 Ci/mmol) were purchased from New England Nuclear (Boston, Mass) and their purity was checked by HPLC with on-line radiochromatographic detection.

Animals and treatment

Timed pregnant Sprague–Dawley rats from Sasco (Omaha, Neb.) arrived on Day 11 of gestation and

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were weighed, randomly placed into three treatment groups and individually housed in clear breeding cages in a temperature controlled nursery with a 14 h light cycle (lights on at 0600 h). Group one ($n = 7$) was administered a liquid diet containing 5% ethanol (w/v), which represents 35% of the daily caloric intake, from Day 12 of gestation until birth (ETOH). Group two ($n = 7$) was weight-matched to group one and pair-fed an isocaloric liquid diet in which sucrose was isocalorically substituted for ethanol during this same period (pair-fed, PF). Group three ($n = 7$) was allowed access to Purina Lab Chow and water *ad libitum* (CHOW). Liquid diets were prepared fresh daily with chocolate Sustacal (Mead Johnson) supplemented with salts (salt mix XIV) and a vitamin diet fortifi-cation mixture (ICN Biochemicals, Cleveland, Ohio).

Newborn rat pups from each group were weighed and sexed by anogenital distance immediately following parturition. Male pups were killed by decapitation and the brains were immediately removed and chilled on ice. The HPOA area was delineated anteriorly by knife cuts 3–4 mm rostral to the optic chiasm, caudal to the mammillary bodies, along the hypothalamic sulci parallel to the central sulcus, and 2–3 mm dorsal to the ventral surface of the tissue block. All tissue samples were immediately frozen on aluminum foil in liquid nitrogen and stored at −70°C until assayed. HPOA tissues from all males within a litter were pooled together and utilized to analyze aromatase and 5α-reductase enzyme activities. Pooling of tissues was necessary to obtain sufficient tissue for duplicate assays using a crude particulate preparation.

Tissue preparation and incubation

Tissue samples from each litter were coded and assayed blind. Tissues were homogenized in Krebs–Ringer phosphate buffer (KRPB, pH 7.2), prepared as previously described [17], at 20 ml/gm. A particulate fraction, which retained full enzyme activity with respect to whole HPOA slices, was prepared by centrifugation of the homogenate at 105,000 *g* (60 min) at 4°C. The resulting pellet was thoroughly washed and resuspended in KRPB at one-half of the original volume and utilized to simultaneously assay both aromatase and 5α-reductase enzyme activities. Incubations were carried out for 2 h at 37°C in 1.0 ml of KRPB containing: 10 mg of the HPOA particulate fraction, 0.5 mmol/l β-NADPH, and 2 μCi [1β-^{3}H]androstenedione. The reaction was initiated with the addition of β-NADPH (37°C) following a 5 min preincubation period at 37°C. Product accumulation was determined in preliminary experiments to be linear with time (up to 3 h) and protein content (up to 4 mg), i.e. well beyond the incubation conditions employed in this study. Control incubations were conducted exactly as described above except HPOA particulate fractions were boiled for 2 min prior to their inclusion in the assay. The reaction was terminated with the addition of 334 μl methanol (25% total methanol) and the incubation vials were capped and briefly stored on ice prior to the solid phase extraction procedure.

Quantitation of aromatase and 5α-reductase reaction products

The enzymatic activity of aromatase was estimated by quantification of the tritium-labeled water released into the incubation media from [1β-^{3}H] androstenedione as follows. Solid phase extraction columns (J. T. Baker, C_{18}) were washed with 2.0 ml of methanol followed by 5.0 ml of deionized double distilled water. The incubation media was percolated through the column followed by 5 column volumes of 25% methanol and this aqueous extract was collected in 20 ml glass scintillation vials. The samples were thoroughly solubilized in 12 ml of scintillation cocktail (ScintiVerse LC, Fisher Scientific, St. Louis, Mo.). Samples were analyzed by liquid scintillation spectrometry (40% counting efficiency) for 10 min or until less than 1% counting error was obtained. The radioactivity in the aqueous extract could not be reduced further either through extraction with ethyl acetate or through the addition of dextran-coated charcoal. This lends support to the assumption that the radioactivity in the aqueous extract was ^{3}H-labeled water. The rate of aromatase activity was calculated using the specific activity of [1β-^{3}H] androstenedione (27.4 Ci/mmol) adjusted for the amount of label in the 1α-position (i.e. 22.0% with lot Nos 2303–297) and the total corrected dpm ^{3}H-labeled water produced. Data was expressed as pmol product formed/2 h/mg protein.

High performance liquid chromatography with on-line radiochromatographic detection was utilized to quantify the amount of (1β-^{3}H)-labeled 5α-androstane-3,17,-dione formed from [1β-^{3}H]androstenedione. Following the elution of ^{3}H-labeled water from the SPE column for the analysis of aromatase activity, the organic fraction was eluted with 5 column volumes of methanol into 15 ml glass conical tubes. Conical tube contents were evaporated under a gentle air stream and concentrated to the tip with benzene. Samples were reconstituted in 40 μl of ethanol and injected into the HPLC. The HPLC system utilized in these studies has been described in detail elsewhere [18]. Raw data from a typical incubation is illustrated in Fig. 1. Clearly, both the substrate and product peaks are baseline resolved. The counting efficiency of on-line radiochromatographic detection (Beckman Instruments Inc., Fullerton, Calif., Model 171) was 20% when scintillation cocktail (ScintiVerse, LC) was mixed with HPLC effluent (3:1 ratio) in a high efficiency mixer. The enzymatic activity of 5α-reductase in neonatal HPOA was similar in preliminary experiments independent of whether we utilized [1β-^{3}H]androstenedione, [1α, 2α-^{3}H]androstenedione or [1,2,6,7,16,17-^{3}H]testosterone as the substrate for the reaction. The rate of 5α-

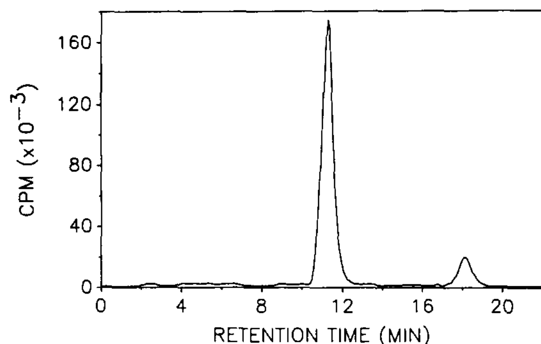

Fig. 1. HPLC separation of androstene-3,17-dione (large substrate peak) and 5α-androstane-3,17-dione (small product peak) using an isocratic acetonitrile–water (50:50, v/v) mobile phase at 1.0 ml/min with a Microsorb ODS (5 μm) 4.6 × 250 mm column. Liquid scintillation cocktail was mixed with the HPLC effluent (3:1, v/v) and the detector response was generated with on-line radiochromatography.

reductase activity was calculated utilizing the specific activity of [1β-^{3}H]androstenedione (27.4 Ci/mmol) and the total corrected dpm within the 5α-androstane-3,17-dione peak. Data was expressed as pmol product formed/2 h/mg protein. Protein content was determined by the method of Bradford [19] using bovine gamma globulin as the standard.

Data analysis

All multiple group data displayed homogeneity of variance and were statistically evaluated by analysis of variance (ANOVA). Duncan's new multiple range test was used to compare differences between individual treatment means. The level of significance was set at $P < 0.05$.

RESULTS

The effects of FAE: general observations

The birth weight (g) of the newborn male pups from each of the three treatment groups was 5.6 ± 0.2 (mean ± SEM, $n = 7$) for the ETOH group, 6.2 ± 0.2 (mean ± SEM, $n = 7$) for the PF group and 6.6 ± 0.2 (mean ± SEM, $n = 7$) for the CHOW group. The birth weight of the FAE male pups was significantly reduced ($P < 0.05$) compared to both the PF and CHOW treatment groups. In addition, the birth weight of the PF group was significantly reduced ($P < 0.05$) compared to the CHOW group suggesting the involvement of a nutritional component (i.e. the effect of feeding a liquid diet) on birth weight. These data are similar to those previously reported [1].

The effects of FAE on 5α-reductase/aromatase activity

The effects of FAE on the enzymatic activities of aromatase and 5α-reductase in the newborn rat HPOA are presented in Table 1. The absence of significant differences ($P > 0.05$) in enzyme specific activity between the ETOH and PF treatment groups suggests that *in utero* exposure to ethanol during Day 12 of gestation through parturition does not influence either 5α-reductase or aromatase enzyme activities. The ratio of 5α-reductase/aromatase enzyme activities also were not significantly different ($P > 0.05$), suggesting that the disposition of androgen within the newborn rat HPOA is not altered by ethanol. The absence of significant differences ($P > 0.05$) between the PF and CHOW treatment groups suggests that the nutritional effects of feeding a liquid diet also have little effect on either 5α-reductase, aromatase or their ratio.

Table 1. Effect of fetal alcohol exposure on hypothalamic-preoptic area (HPOA) 5α-reductase and aromatase enzyme activities[a]

HPOA enzyme	ETOH group	PF group	CHOW group
5α-Reductase	4.76 ± 0.19	4.69 ± 0.24	5.18 ± 0.22
Aromatase	0.40 ± 0.03	0.39 ± 0.03	0.45 ± 0.02

[a] Data are expressed as pmol product formed/2 h/mg protein (mean ± SEM, $n = 7$). The designations ETOH, PF and CHOW represent the fetal alcohol exposed, pair-fed and chow-fed animal treatment groups, respectively.

DISCUSSION

The local conversion of circulating androgens within the rat HPOA either through the 5α-reductase pathway to the more potent 5α-reduced androgens or through the aromatase pathway to estrogens is known to be essential for the normal sexual differentiation of the CNS [11–16]. FAE during the final week of gestation in the rat permanently induces both structural [1–4] and functional [5–8] alterations in the rat CNS. We have previously demonstrated that FAE inhibits testicular steroidogenesis in newborn rats accounting for the observed decline in plasma testosterone levels [9]. This reduction in androgen substrate available to the developing rat HPOA is one possible mechanism by which FAE may alter the sexual differentiation of the CNS. In the present study, we have investigated another possible mechanism by which FAE may alter this developmental process, namely, altered androgen metabolism within the newborn rat HPOA. The results indicate that neither HPOA 5α-reductase activity, aromatase activity or their ratio are influenced by FAE. These data support the hypothesis that FAE alters the sexual differentiation of the CNS by reducing the availability of androgens to the developing brain via a lesion in androgen biosynthesis at the level of the testis.

Our data appear to be in contrast to those of McGivern *et al.* [20] who demonstrated a slight increase in the male but not female HPOA aromatase activity following *in utero* ethanol exposure. These differences may be the result of different tissue preparations used to analyze aromatase activity in the rat HPOA. We utilized a washed particulate fraction which presumably contains less contaminating levels of endogenous steroids than a whole homogenate preparation. An artefactual increase in aromatase activity might be realized using whole homogenate

preparations if the hypothalamic androgen content was significantly reduced following prenatal ethanol exposure. This is likely to be the case since plasma androgen levels are known to be significantly reduced following FAE [1–3, 21].

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