Journal of Steroid Biochemistry, Vol. 9, pp. 443–447

0022-4731/78/0501-0443$02.00/0

QUANTITATIVE EVALUATION OF CYTOSOL AND NUCLEAR [^{3}H]-ESTRADIOL SPECIFIC BINDING IN THE FETAL BRAIN OF GUINEA PIG DURING FETAL ONTOGENESIS

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SUMMARY

Specific [^{3}H]-estradiol binding to macromolecules was found in the cytosol and nuclear extracts of fetal guinea pig brain from at least 29 days of gestation. The nuclear extracts were obtained by successive extractions with (A) 0.1 M Tris HCl–0.0015 M EDTA (0.1 M Tris); (B) 0.3 M NaCl–0.1 M Tris HCl (0.3 M NaCl), and (C) 1 M NaCl–0.01 M Tris HCl (1 M NaCl). The binding affinity of estradiol in the cytosol fraction has a dissociation constant (K_D) of 3.2×10^{-10} M. The specific binding of estradiol in the cytosol increases throughout fetal evolution and the average values of 3 experiments are 10.5 fmol/mg DNA at 29–35 days of gestation, 52 fmol at 37–38 days, 72.1 fmol at 44–45 days, 137 fmol at 49–50 days and 187 fmol at 62–65 days. After birth these values are 161 fmol in newborns (<24 h, 65 fmol at 1 week, 53 fmol at 4 weeks and 66 fmol in the adult. On the other hand, the specific binding in the combined nuclear extracts (0.1 M Tris + 0.3 M NaCl + 1 M NaCl) remains at low values which are respectively (in fmol/mg DNA): 9.5, 4.8, 5.3, 5.8, 7.2, 6.3, 6.2, 3.0, and 12. Most of this specific binding (50–70% of the total nuclear binding) in the nuclei is localized in the 1 M NaCl extracts. Studies of specific [^{3}H]-estradiol binding in the cytosol fraction which were carried out at 35–36, and 60–65 days of gestation in female and male fetuses did not present significant differences between males and females.

INTRODUCTION

The selective uptake and accumulation of estrogens in the rat brain was demonstrated after administration of labelled estradiol [1] or hexestrol [2]. Similarly in guinea pig and hamster brains an increase was observed in the ratio of tissue versus plasma concentration of radioactivity with time, after subcutaneous injection of [^{3}H]-estradiol [3]. Eisenfeld[4] found specific binding of estradiol in the cytosol fraction of the adult rat brain which was localized mainly in the hypothalamus, with significant quantities being found in the cerebrum. Estradiol receptors have also been found in adult mouse brain [5] and a specific estradiol-binding substance has been detected in neonatal rat brains [6]. In this laboratory the presence of cytosol and nuclear specific [^{3}H]-estradiol binding in the fetal brain of guinea pig was presented in a preliminary note [7]. Consequently it was interesting to study the binding of [^{3}H]-estradiol in the brain during the fetal period and to follow its variation during fetal development of the guinea pig which is the subject of the present paper.

MATERIALS AND METHODS

Biological material. Brains of Hartley Albino guinea pig fetuses were used. Fetal age was established with an error of ±24 h since the female guinea pigs were mated for only 24 h. The brain of newborns, immature and adult female guinea pigs were also used.

Radioactive material. [6,7-^{3}H]-Estradiol (S.A. 47.9 Ci/mmol) was purchased from NEN Chemicals GmbH., (Frankfurt, West Germany). Purity was controlled by paper chromatography in the system: isooctane–methanol–water (1:4:3:2, by vol) and, after acetylation in the system: isooctane–methanol–water (5:3:2, by vol). Purity was 98%.

Experimental conditions. The fetuses obtained at different periods of gestation were decapitated and the brains were removed, taking all cerebral tissue anterior to the mammillary bodies. Cell suspensions from 2 g of the fetal brain were prepared at 2°C by very lightly homogenizing the tissue which was then incubated in 5×10^{-8} M [^{3}H]-estradiol without or with a 100–300 fold excess of unlabelled estradiol. The incubations were carried out in Krebs–Henseleit buffer [8] pH 7.4 at 37°C for 15 min in the proportions of 1 g tissue to 2 ml of buffer. In addition, in some experiments using fetuses of the same age, the cytosol fraction of the brain was isolated after homogenization in 0.01 Tris, HCl–0.0015 M EDTA and this fraction was incubated in 4.14×10^{-9} M [^{3}H]-estradiol without or with a 100 fold excess of unlabelled estradiol. (The details of other experimental conditions are indicated in the results section or in the legends of tables or figures.)

Cell fractionation. The tissues were fractionated according to the method of Chauveau *et al.*[9] with the modifications indicated previously [10]. The tissues were homogenized in 0.25 M sucrose–0.01 M Tris, HCl–0.003 M $CaCl_2$ (pH 7.4) and centrifuged at 900 *g*. The 900 *g* supernatant was centrifuged at 200,000 *g* to separate the soluble cytosol fraction from

the mitochondria–microsomal pellet. The 900 *g* pellet was washed with 0.4 M sucrose–0.01 M Tris, HCl–0.003 M $CaCl_2$ and centrifuged at 900 *g*. This 900 *g* pellet was homogenized in 1.8 M sucrose–0.01 M Tris, HCl–0.003 M $CaCl_2$, layered on an equal volume of the same solution and centrifuged at 200,000 *g* to isolate purified nuclei. All of these steps were carried out at 2°C. The RNA/DNA ratio in the brain nuclei (fetuses of 50–55 days of gestation) isolated by this method was 0.257 ± 0.06 (S.D.) and the nuclear protein/DNA ratio, 2.791 ± 0.7 (S.D.). The purified nuclei were extracted successively with the following solutions:

(A) 0.1 M Tris, HCl–0.0015 M EDTA, pH 7.4 (0.1 M Tris); (B) 0.3 M NaCl–0.01 M Tris HCl, pH 7.4 (0.3 M NaCl); (C) 1 M NaCl–0.01 M Tris HCl, pH 7.4 (1 M NaCl); (D) 3 M NaCl–0.01 M Tris HCl, pH 7.4 (3 M NaCl pH 7.4); (E) 3 M NaCl–0.01 M Tris HCl, pH 8.4 (3 M NaCl pH 8.4); (F) 0.2 N HCl; (G) 0.2 N NaOH; (H) 90% ethanol (v/v).

Determination of specific binding. The tissues were incubated either with [^{3}H]-estradiol alone or with a 100–300 fold excess of unlabelled estradiol. Specific binding was calculated by the difference in binding between these two incubations as determined either by Sephadex G-15 column chromatography at 4°C or by dextran-coated charcoal using 0.5% charcoal and 0.05% w/v dextran [11]. Equilibrium constants were measured by the Scatchard method [12] with the graphical correction of Rosenthal[13] to correct for non-specific binding (Details of the experimental conditions are indicated in the results section).

Protein and DNA assays. The method of Lowry *et al.*[14] was used to measure protein concentrations and the assay of Burton[15] for DNA.

Radioactivity measurement. Radioactivity in aqueous solutions was measured in Instagel (Packard, Inc.) and radioactivity in organic solvents in POPOP-PPO-toluene scintillation solution.

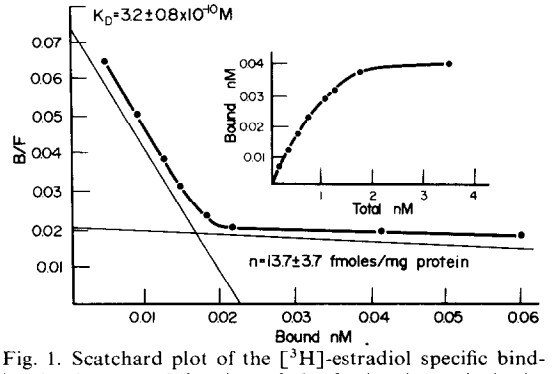

Fig. 1. Scatchard plot of the [^{3}H]-estradiol specific binding in the cytosol fraction of the fetal guinea pig brain. Brain cytosols of guinea pig (50–55 days of gestation) (containing 2.2 mg of protein/ml) were incubated with various concentration of [^{3}H]-estradiol (1.12–37 × 10^{-10} M) in the absence or presence of 5.8 × 10^{-7} M unlabelled estradiol for 4 h at 0°C. Binding was determined by charcoal adsorption. The calculation of the dissociation constant (K_D) and the number of sites (n) was obtained with the values of 7 determinations.

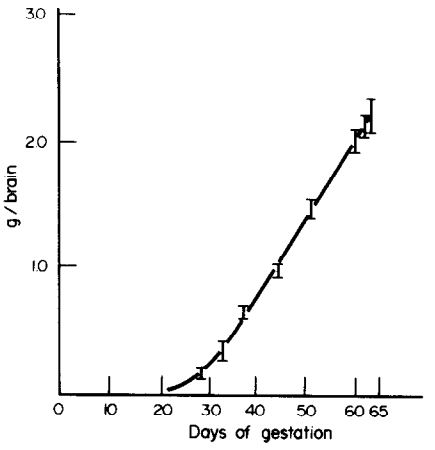

Fig. 2. Weight of guinea pig brain during fetal development. The values represent the average of 30 fetuses at 29–35 days, 18 at 37–38 days, 12 at 44–45 days, 12 at 49–50 days, and 6 at 62–65 days of gestation.

RESULTS

(1) *Affinity constant of the* [^{3}H]-*estradiol receptor complex in the cytosol fraction of the fetal brain*

The affinity of [^{3}H]-estradiol–macromolecule complexes was studied in the cytosol fraction according to the Scatchard Method; the results are indicated in Fig. 1. As is observed two kinds of populations are obtained, one with high affinity, $K_D = 3.2 \pm 0.8 \times 10^{-10}$ M and a number of binding sites n of 13.7 ± 3.7 fmol/mg protein and another population with a low affinity.

(2) *Specific* [^{3}H]-*estradiol binding in the cytosol and nuclei of the brain during fetal development*

In order to correlate the growth of the fetal brain and the quantity of specific estradiol receptors, the weight of the brain during fetal evolution was measured and the data are indicated in Fig. 2. Also, a study was carried out to establish the variation of nuclear protein and DNA (per g of wet tissue) in the fetal brain during fetal development, these data are indicated in Fig. 3. This figure also includes the data on nuclear DNA and proteins obtained in newborns, immature and adult females. As is observed, the nuclear concentrations of protein and DNA (per g tissue) at the early stages of gestation (29–35 days) are 2–3 times greater compared to the end of gestation. In successive nuclear extractions (see material and methods), 80–90% of the nuclear DNA is extracted by the 1 M NaCl solution, 2–7% by the 3 M NaCl (pH 7.4), 0.1–3% by 0.3 M NaCl (pH 7.4) and the remainder by 3 M NaCl (pH 8.4) 0.2 N HCl, 0.2 N NaOH and 90% ethanol v/v.

The specific binding of [^{3}H]-estradiol in the cytosol and in the different nuclear extracts of the fetal brain during fetal evolution is indicated in Table 1. As is observed in this table most of the specific [^{3}H]-estra-

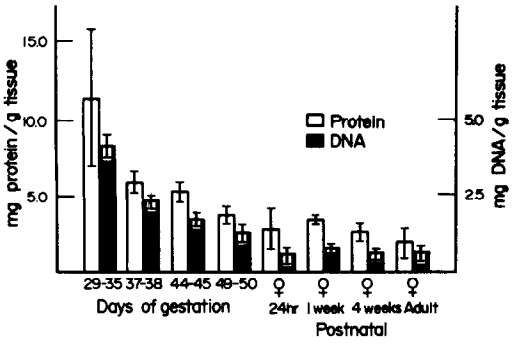

Fig. 3. Concentration of nuclear protein and DNA in fetal brain during development. The bars represent the average plus the extreme values of 2–4 experiments.

diol binding is found in the cytosol fraction and this value increases significantly throughout fetal development, maximum values are found at the end of gestation. Specific binding in the nuclei is localized mainly in the 1 M NaCl nuclear extracts and the values in this nuclear fraction and in the others are relatively low throughout gestation. Figure 4 gives the specific binding of [^{3}H]-estradiol per mg of DNA in the cytosol and in the fetal nuclei at different steps of development. Of special interest is the ratio of specific binding in the cytosol to the binding in the nuclei; this ratio is close to one at 29–35 days of gestation and increases many times at the end of gestation.

In Table 2 is indicated the specific [^{3}H]-estradiol binding after incubation of the cytosol fraction of fetal male and female brains during fetal development. It can be observed that these values are similar to those obtained in the same cytosol fractions in which [^{3}H]-estradiol was incubated with the total cell suspension. Furthermore in the 2 gestation periods studied (35–36 and 60–65 days) slightly higher values of specific [^{3}H]-estradiol binding were found in the cytosol fraction of fetal females than in males. It should be noted that the values of specific [^{3}H]-estradiol binding were similar whether binding was determined by Sephadex G-15 chromatography or by charcoal-dextran adsorption.

DISCUSSION

The present study shows the presence of specific [^{3}H]-estradiol binding in the cytosol and nuclei of fetal guinea pig brain from at least 29 days of gestation. At that period of fetal evolution the quantity of specific [^{3}H]-estradiol binding is low in both the

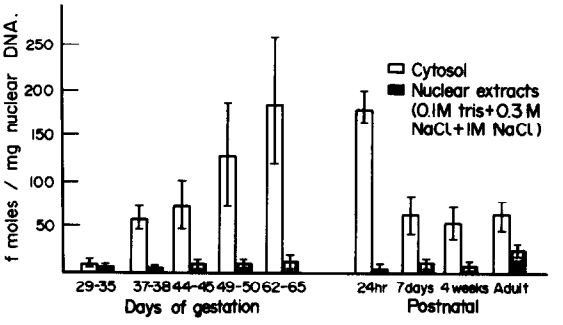

Fig. 4. Specific [^{3}H]-estradiol binding in guinea pig brain during development. 2 g Of brain were incubated with 5.2×10^{-8} M [^{3}H]-estradiol in Krebs–Henseleit buffer at 37°C for 15 min or with a 100–300 fold excess of unlabelled estradiol. The quantity of specifically bound [^{3}H]-estradiol in the cytosol and in the combined 0.1 M Tris, 0.3 M NaCl and 1 M NaCl nuclear extracts is expressed as fmol per mg of nuclear DNA. The bars represent the extreme values of 2–3 experiments with 22 fetuses at 29–35 days, 9 at 37–38 days, 5 at 44–45 days, 6 at 49–50 days and 3 at 62–65 days of gestation.

Table 1. Cytosol and nuclear specific binding of [^{3}H]-estradiol in fetal brain of guinea pig during fetal development after incubation of the whole cell

Days of gestation	Cytosol		Nuclear extracts					
			0.1 M Tris		0.3 M NaCl		1 M NaCl	
	(fmol/ mg protein)	(fmol/g tissue)	(fmol/ mg protein)	(fmol/g tissue)	(fmol/ mg protein)	(fmol/g tissue)	(fmol/ mg protein)	(fmol/g tissue)
29–35	3.5	43	6.0	5.0	7.0	7.0	1.0	27.0
37–38	8.5	140	17.0	6.1	11.5	4.3	3.4	2.0
44–45	7.0	117	2.8	4.1	3.4	2.0	0.6	2.4
49–50	15.5	174	4.8	2.1	6.1	1.0	2.3	3.9
62–65	13.5	283	3.5	1.6	6.1	2.5	4.0	6.6
Female <24h	14.5	113	N.D.	N.D.	N.D.	N.D.	0.6	3.0
Female 1 week	8.0	52	N.D.	N.D.	N.D.	N.D.	1.5	5.5
Female 4 weeks	3	32	N.D.	N.D.	N.D.	N.D.	0.5	1.6
Female Adult	9.0	48	10.0	1.7	20.0	5.0	6.5	1.7

2 g Of tissue were incubated with 5.2×10^{-8} M [^{3}H]-estradiol or with the same quantity of radioactivity plus a 100–300 fold excess of unlabelled estradiol in Krebs–Henseleit buffer at 37°C for 15 min. The values represent the average of 3 experiments using 22 fetuses at 29–35 days, 18 at 37–38 days, 5 at 44–45 days, 6 at 50 days and 3 at 62–65 days. N.D. = not detectable.

Table 2. Cytosol specific binding of [^{3}H]-estradiol in male and female fetal brains of guinea pig (in fmol/mg protein)

Days of gestation	Female fetuses	Male fetuses
35–36	7.16 ± 1.83 S.D.	6.0 ± 1.41 S.D.
60–65	13.00 ± 1.58 S.D.	9.5 ± 3.87 S.D.

The cytosol fractions of the fetal brains containing 2–3 mg of protein/ml were incubated with 4.14×10^{-9} M [^{3}H]-estradiol without or with a 100 fold excess of unlabelled estradiol. The incubations were carried out in Tris HCl buffer solution at 4°C for 4 h. ($n = 5$ determinations.)

cytosol and nuclear fractions but binding in the cytosol increases significantly during fetal evolution. On the other hand the total specific binding in the nuclear extracts obtained by 0.1 M Tris–HCl, 0.3 M NaCl, and 1 M NaCl solutions remains low throughout fetal development. After birth, in newborn animals, the cytosol specific [^{3}H]-estradiol binding is still high but decreases in immature and adult animals.

As is indicated in Table 1, most of the specific [^{3}H]-estradiol binding sites in the nuclei are localized in the fraction extracted with 1 M NaCl, which contains 80–90% of the nuclear DNA. Similar data of "resistant nuclear sites" were found for the [^{3}H]-estradiol receptor in other fetal tissues of guinea pig (kidney, lung, and uterus) [10, 16, 17]. Steroid hormone receptors resistant to extraction with 0.3–0.4 M NaCl or KCl were also recently found for androgens in the epididymis of castrated rabbits [18] and for estrogens in the uterus of immature rats [19].

Specific binding of [^{3}H]-estradiol in the fetal brain is not due to plasma contamination because under the same experimental conditions the fetal plasma of guinea pig does not bind (or binds very little) [^{3}H]-estradiol [17]. Furthermore in recent studies in this laboratory, the fetal plasma was incubated with [^{3}H]-estradiol in the same experimental conditions (temperature, time, protein and tritiated estradiol concentrations) used in the incubation of the cytosol fraction of the fetal brain with [^{3}H]-estradiol. The results show that in the very small percentage of [^{3}H]-estradiol bound to fetal plasma proteins (less than 1% of the incubated radioactivity) a 100 fold excess of unlabelled estradiol does not decrease the amount of [^{3}H]-estradiol in the plasma [^{3}H]-estradiol macromolecule complexes (unpublished data).

Comparative data on the evolution of estradiol receptors in other fetal tissues (kidney, lung and uterus) [20] of the same animal species indicate that in contrast to the present data the nuclear receptors in these tissues increase with fetal age and decrease after birth. Complementary studies concerning the number of available receptor sites using the nuclear exchange method as well as the evaluation of estrogen concentrations in the fetal brain could provide a solution to this problem. These studies are now in progress.

In another series of studies to determine the comparative values of specific [^{3}H]-estradiol binding sites in female and male fetuses, the cytosol fraction of these tissues was incubated with [^{3}H]-estradiol and the data shown in Table 2 indicate that the values (per mg protein) are of the same order as those obtained in the cytosol fraction when whole tissue was incubated. At the 2 ages studied, 35–36, and 60–65 days of gestation the specific binding of [^{3}H]-estradiol in the cytosol of female animals was slightly higher than in males. Complementary studies on the determination of specific binding at other periods of gestation as well as the localization in the different areas of the brain throughout fetal development could provide valuable information for the physiological interpretation of these differences. The values of specific binding of [^{3}H]-estradiol found in the cytosol fraction of fetal guinea pig brain are of the same order of magnitude as those found by Attardi and Ohno[21] in very young mice (3–23 days old). These authors also did not find significant differences in the quantity of [^{3}H]-estradiol specific binding in the brain of female and male animals.

It is interesting to note that similar specific [^{3}H]-estradiol binding was also found in cerebral cortex of adult guinea pigs [22] but recently Plapinger *et al.*[23] suggested that specific estradiol binding was present in the hypothalamus-preoptic area-amygdala in both fetal and adult guinea pigs but not on the cerebral cortex. These contradictions remain to be clarified and it would be particularly important to know if these specific binding sites are involved in some biological function during intrauterine life. However in spite of these contradictions the data for the affinity of specific [^{3}H]-estradiol binding found in the present paper ($K_D = 3.2 \times 10^{-10}$ M) are similar to those found for the classical estradiol receptor in uterine tissues [24]. Furthermore the analysis in sucrose density gradients shows in both male and female fetal guinea pig brain cytosol (60–65 days of gestation) the presence of a component with a coefficient of sedimentation of 7–8S [7 and unpublished observations]. Furthermore, in agreement with the presence of specific binding sites in the fetal brain of guinea pig, recent studies in this laboratory using the autoradiographic method have demonstrated the localization and selective uptake of the radioactivity in the fetal brain 30 min after *in vivo* and *in situ* injection of estradiol to the fetus [25]. Studies are in progress to compare the presence of this component in other periods of gestation as well as in newborn and adult animals.

Finally the fact that very little estradiol is bound to fetal plasma protein in this species raises the question of how the fetus is protected against the biological action of estradiol. This could be explained by the intense metabolism of this hormone to inactive metabolites which has been demonstrated after *in vivo* and *in situ* administration of [^{3}H]-estradiol to the fetus. Most of the circulating radioactive material (65–75%) is in the form of sulphates of estradiol and

estrone [17]. Protection could also be carried out by binding to non specific sites which is very significant in the cytosol fraction of different fetal tissues (brain, kidney, lung) [17, 20]. The quantitative evaluation of estrogens during fetal development in plasma and in different fetal tissues can also give interesting information to explain the mechanism of protection.

Acknowledgements—Part of the expenses of this work was defrayed by a grant from the Centre National de la Recherche Scientifique (C.N.R.S.) France (Equipe de Recherche C.N.R.S. No. 187) and by a grant from the Institute National de la Sante et de la Recherche Medicale (INSERM) France (ATP-6-74-27).

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