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CALORIMETRIC APPROACH TO THE STUDY OF 5α-DIHYDROTESTOSTERONE AND ESTRADIOL RECEPTORS IN RAT HYPOTHALAMUS CYTOSOL

J. KNIEWALD, S. ČALA, P. MILDNER and Z. KNIEWALD
Laboratory of Biochemistry, Technological Faculty, University of Zagreb, 41000 Zagreb, Yugoslavia

SUMMARY

The hypothalamus and pituitary are the sites through which androgen and estrogen hormones perform the feedback control of gonadotropic mechanism. The microcalorimetric method was used to study the specific reaction between 5α-dihydrosterone (5α-DHT) and Estradiol (E_2) and their receptors in the hypothalamus of male and female rats at the age of 28 d. In order to compare binding affinity, experiments were performed at 4° and 37°C. Molar enthalpy changes for E_2 at 37°C are -43.96 ± 0.453 kcal/mol for female and -25.04 ± 0.222 kcal/mol for male rats, respectively. At 4°C molar enthalpy changes are approximately 50% higher for female and 25% higher for male rats. For 5α-DHT molar enthalpy changes at 37°C are -26.67 ± 1.221 kcal/mol for female and -18.60 ± 0.512 kcal/mol for male rats. At 4°C increase for female rats is over 60% and for male rats 40%. The effect of sex difference is much better expressed for E_2 than for 5α-DHT. Possible binding of E_2 and 5α-DHT to hypothalamus receptors in the female and male rats could be in some connection with the mechanism in regulation of gonadotropic feedback system. Ratios of molar enthalpy changes for E_2: 5α-DHT are 2:1 in females and 1:1 in males, at 4°C. Difference between these ratios could be an answer for the physiological sex difference.

INTRODUCTION

Microcalorimetric method enabled an approach to the study of interaction of hormones and proteins [1]. Such investigations allow more extensive observation of protein–hormone interactions from the beginning of complex formation. The interaction between various hormones and receptors from rat prostate cytosol was studied [2]. It was found that the affinity of cytosol receptors for 5α-dihydrotestosterone and progesterone is far greater than for testosterone. Testosterone binding to receptor was endothermic, and this finding brings into question the receptor–testosterone complex formation [2].

The anterior hypothalamus and pituitary are considered to be the sites through which androgen and estrogen hormones perform the feedback control of gonadotropic mechanism. There is no doubt that various regions of the hypothalamus contain estrogen receptors. Both anterior, mid and posterior portions of the hypothalamus contain 8–10 S cytoplasmic receptors for estradiol (E_2) [3–5] and there is general agreement that E_2 uptake by unfractionated hypothalami from male and female rats is similar [3–8]. There is much less information about the presence of an androgen-binding protein in the rat brain. Few data about receptors are accumulated on the anterior pituitary and the hypothalamus [9]. Kato and Onouchi[10] have shown the existence of a receptor for 5α-dihydrotestosterone (5α-DHT) in the hypothalamic cytosol of male rats. 5α-DHT binding component has a sedimentation coefficient of 8.6 S and the dissociation constant of the androgen–protein complex is 7.4×10^{-10} M [10].

In the present study, the microcalorimetric method was used to study the specific reaction between hormones (5α-DHT and E_2) and their receptors in rat hypothalamus. We performed our experiments at 4° and 37°C, so we were able to compare approximately the binding affinity using microcalorimetric measurements with results obtained during sucrose density gradient resolution. We have found that the binding reactions at two different temperatures were exothermic, using basal hypothalamus of male and female rats at the average age of 28 days. In addition to microcalorimetric measurements, in the present paper we demonstrated new evidence for the existence of receptors in basal rat hypothalamus of male and female rats, for the above mentioned hormones. We applied separation in sucrose density gradient, in order to obtain the sedimentation coefficient for hormone–receptor complex.

EXPERIMENTAL

Chemicals

Radioactive steroids, [1,2,4,5,6,7-^{3}H]-5α-dihydrotestosterone, 175 Ci mmol^{-1} and [6,7-^{3}H]-Estradiol, 41 Ci mmol^{-1}, were obtained from the Radiochemical Centre (Amersham, U.K.) and purified by t.l.c. before use. The following unlabelled steroids were used without further purification: E_2 from Vismara (Como, Italy) and 5α-DHT from Steraloids Inc. Bovine serum albumin Fraction V (BSA) was purchased from BDH Biochemicals Ltd. and pig heart fumarase was purchased from Boehringer Mannheim GmbH. All other chemicals employed were commercial preparations of analytical grade.

Tissue sampling

Male and female rats of the age of 25–30 days (Fisher strain) were decapitated and immediately the whole hypothalamus, including the preoptic area, mammillary body and a part limited by a 2 mm cut from the midline, was removed as a block. The tissues from usually 40 rats were collected and thoroughly rinsed in ice-cold 10 mM Tris–HCl buffer, pH 7.4, containing 1.5 mM EDTA and 2 mM 2-mercaptoethanol to remove traces of blood. Tissues were homogenized and the homogenate was then centrifuged at 105,000 *g* for 30 min in a Beckman ultracentrifuge Model L-3-50, rotor SW 41.

Microcalorimetric measurements

By microcalorimetric determinations we checked the heat involved in a hormone–hypothalamus receptor complex formation. Microcalorimetric measurements were carried out with a Model 10700-1 LKB Microcalorimeter, using an 18-carat golden reaction channel, designed by Monk *et al.*[11]. Approximately equal vol. of hormone and protein solutions were mixed, and the flow rate was about 0.0033 ml/s and the steady-state heat flux ranged from 0.1 to 15 μcal/s. The hormone stock solutions of unlabelled hormones were prepared in absolute ethanol and then diluted in 10 mM Tris–HCl buffer, pH 7.4; the final concentration of ethanol was 10 μl/ml. It was important, therefore, to perform all dilution experiments with a buffer containing the same amount of ethanol. The heats of dilution for each hormone and hypothalamus cytosol used in the experiments were measured separately and then subtracted from the measured heat of mixing to obtain the heat of reaction. Hormone solutions for microcalorimetry were prepared in 10 mM Tris-HCl buffer pH 7.4, on the day of experiments in the following concentration ranges: 34.4 to 103.2 μmol for 5α-DHT and 9.2 to 55.1 μmol for E_2, respectively. The cytosol was prepared from the rat hypothalami by dilution in 10 mM Tris–HCl buffer pH 7.4, and the protein concentration in cytosol was adjusted to be 1 mg/ml and was kept constant in all experiments.

The heat effect due to mixing of reactants (W_m) has two components; one resulting from the heats of reaction (W_r) and another resulting from the heats of dilution of the components of the two solutions (W_d). So, $W_r = W_m - W_d$, and the heats of dilution are determined in separate experiments. Enthalpy of reaction is calculated from the equation: $\Delta H' = W_r/f.\ \Delta R$, where $\Delta H'$ is the molar enthalpy change for the reaction (kcal/mol), f is the flow rate of the resulting solution (ml/s) and ΔR is the change in the concentration of the limiting reactant R (mol). By using this equation the molar enthalpy change in all our experiments was calculated.

Sucrose density gradient separation

The supernatant fraction (105,000 *g*) of the hypothalamus tissue (0.2 ml cytosol) was incubated with gentle shaking for 30 min at 4° or 37°C with 4.7×10^{-13} mol of purified [^{3}H]-DHT or with 5.9×10^{-13} mol of purified [^{3}H]-E_2 dissolved in 0.1 ml of 10 mM Tris–HCl buffer pH 7.4. Separation in sucrose density gradient (5–20%) was performed by ultracentrifugation for 18 h at 180,000 *g* at 4°C. The fractions were measured for radioactivity in Mark II Nuclear Chicago liquid scintillation counter using Permablend TMIII scintillant. Correction for quenching in all samples was made by the internal standard method. Apparent sedimentation coefficients (S) were determined by the use of BSA (4.6 S) and pig heart fumarase (8.5 S) as reference standards according to the method of Martin and Ames[12].

RESULTS

Tables 1 and 2 show the dependence between the heat of binding and the concentration of E_2 and 5α-DHT in 10 mM Tris–HCl buffer pH 7.4 at 4°C and 37°C, respectively. Results are presented for binding of hormones to cytosol receptors from hypothalami of male and female rats at the age of 28 days. The amount of hormone was sufficient to saturate completely the cytosol solutions in the concentration range used, as indicated by the straight-line fits of the data, when the observed heat of binding W is plotted vs the hormone concentration.

As shown in Table 1 molar enthalpy changes calculated for E_2 binding with hypothalamus cytosol receptors from female rats are -43.96 ± 0.453 kcal/mol and from male rats -25.04 ± 0.222 kcal/mol, at 37°C, respectively. Molar enthalpy changes for binding of E_2 to male and female receptors in the hypothalamus are significantly higher at 4°C in comparison with the results at 37°C. For 5α-DHT binding to cytosol receptors from hypothalami at 4°C and 37°C, molar enthalpy changes are presented in Table 2. Binding of 5α-DHT to cytosol receptors from females gives values of molar enthalpy change of -26.67 ± 1.221 kcal/mol and in the case of receptors from males -18.60 ± 0.512 kcal/mol, at 37°C, respectively. Experiments at 4°C give values for molar enthalpy changes of -37.23 ± 0.411 kcal/mol for 5α-DHT binding to receptors from females and -30.31 ± 0.379 kcal/mol when 5α-DHT binds to hypothalami receptors from males.

Sucrose density gradient pattern of the hypothalamic cytosol from male and female rats following *in vitro* administration of [^{3}H]-E_2, is shown in Fig. 1. Binding of E_2 by hypothalamic cytosol receptor, with sedimentation coefficient of 7 S, might be observed only if the binding procedure is performed at 4°C. When cytosol fractions were incubated *in vitro* [^{3}H]-E_2 at 37°C in the same period of time, radioactivity peak in the 7 S region was hardly detectable.

If the hypothalamic cytosol is incubated with [^{3}H]-E_2 (5.9×10^{-13} mol/0.3 ml of incubation media) in the presence of unlabelled E_2 (1000 fold higher conc.), binding of [^{3}H]-E_2 is completely abolished.

Table 1. Heat of binding of estradiol to hypothalamus cytosol from the female (F) and the male (M) rats at the age of 28 days, in 10 mM Tris–HCl buffer, pH 7.4

Temperature °C	Rats	Estradiol concentration (mol/s × 10^9)	Observed heat effect[1] (kcal/s × 10^9) W ± SD[2]	Molar enthalpy change[3] (kcal/mol of estradiol) ΔH′ ± SD[2]
4	F	0.0306	−2.31 ± 0.064	
4	F	0.0611	−4.15 ± 0.136	−67.49 ± 0.518
4	F	0.1222	−8.31 ± 0.432	
37	F	0.0306	−1.63 ± 0.080	
37	F	0.0611	−2.96 ± 0.194	−43.96 ± 0.453
37	F	0.1222	−5.44 ± 0.237	
4	M	0.0611	−1.90 ± 0.017	
4	M	0.1222	−3.84 ± 0.253	−31.25 ± 0.176
4	M	0.1833	−5.71 ± 0.371	
37	M	0.0611	−1.21 ± 0.034	
37	M	0.1222	−2.90 ± 0.096	−25.04 ± 0.222
37	M	0.1833	−4.53 ± 0.259	

[1] Three to 5 measurements were made for each concentration.
[2] Deviation from observed experimental values.
[3] Molar enthalpy change observed at protein concentration of 1 mg/ml in the rat hypothalamus cytosol.

Using the sucrose density gradient resolution we studied 5α-DHT binding to the hypothalamic cytosol from male and female rats, as shown in Fig. 2. There is a great difference between the sedimentation pattern obtained by hypothalamic cytosol from male and female animals. Obviously, the amount of bound radioactivity in the cytosol fraction was considerably higher in hypothalamus taken from female than from male rats. Further, there was observed quite a difference in sedimentation coefficients for [^{3}H]-DHT receptors. The hypothalamic cytosol from male rats possessed the receptor with sedimentation coefficient of 6.5 S and from female 7.5 S (see Fig. 2). With raising temperature of incubation, the peak of radioactivity was decreased, similar to our observation with the binding of E_2 to hypothalamic receptors.

DISCUSSION

As shown by Kato and Onouchi[10] the cytosol of the hypothalamus of male rats possesses a macromolecular component, "receptor", capable of binding 5α-DHT with a high affinity. Since our results from sucrose gradient pattern of male as well as female rats reveal the presence of receptor's sites for E_2 and 5α-DHT, our aim was to determine the total heat change occurring during the hormone–receptor complex formation. We found, in all cases, that the reactions between E_2 or 5α-DHT and hypothalamic receptors are exothermic (see Tables 1 and 2).

As shown in Table 1 the molar enthalpy change for E_2 is 116% higher for binding to female receptors than for males at 4°C. These results are in good corre-

Table 2. Heat of binding of 5α-dihydrotesterone to hypothalamus cytosol from the female (F) and the male (M) rats at the age of 28 days, in 10 mM Tris–HCl buffer, pH 7.4

Temperature °C	Rats	5α-dihydrotestosterone concentration (mol/s × 10^9)	Observed heat effect[1] (kcal/s × 10^9) W ± SD[2]	Molar enthalpy change[3] (kcal/mol of 5α-dihydrotestosterone) ΔH′ ± SD[2]
4	F	0.1156	−4.81 ± 0.209	
4	F	0.2312	−8.72 ± 0.642	−37.23 ± 0.411
4	F	0.3468	−12.91 ± 0.565	
37	F	0.1156	−3.38 ± 0.284	
37	F	0.2312	−5.68 ± 0.134	−26.67 ± 1.221
37	F	0.3468	−9.06 ± 0.709	
4	M	0.1156	−3.91 ± 0.092	
4	M	0.2312	−7.01 ± 0.117	−30.31 ± 0.379
4	M	0.3468	−10.65 ± 0.328	
37	M	0.1156	−1.45 ± 0.017	
37	M	0.2312	−4.11 ± 0.126	−18.60 ± 0.512
37	M	0.3468	−6.28 ± 0.274	

[1] Three to 5 measurements were made for each concentration.
[2] Deviation from observed experimental values.
[3] Molar enthalpy change observed at protein concentration of 1 mg/ml in the rat hypothalamus cytosol.

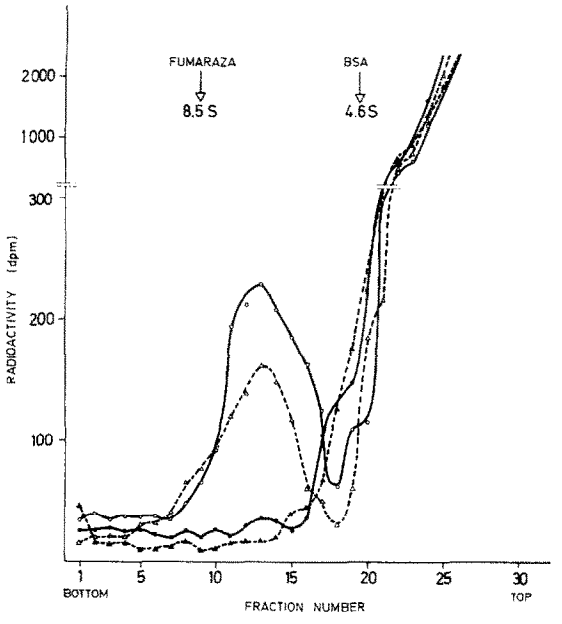

Fig. 1. Sucrose density gradient patttern of the cytosol from hypothalamus. The hypothalamic cytosol from female rats of the age of 28 days (0.2 ml containing 1.38 mg of protein) was incubated with [6,7-^{3}H]-estradiol (1.6×10^{-9} M) for 30 min at 4°C (○—○) and for 30 min at 37°C (●—●). The hypothalamic cytosol from male rats of the age of 25 days (0.2 ml containing 1.34 mg of protein) was incubated with [6,7-^{3}H]-estradiol in the same concentration for 30 min at 4°C (△—△) and for 30 min at 37°C (▲—▲).

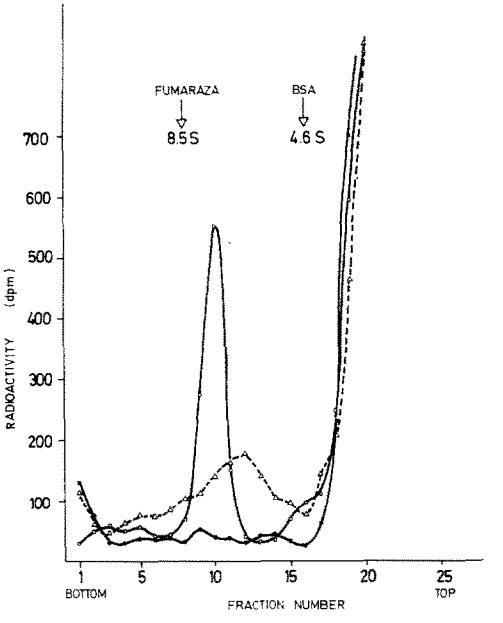

Fig. 2. Sucrose density gradient pattern of the cytosol from hypothalamus. The hypothalamic cytosol from female rats of the age of 28 days (0.2 ml containing 1.18 mg of protein) was incubated with [1,2,4,5,6,7,-^{3}H]-5α-dihydrotestosterone (1.2×10^{-9} M) for 30 min at 4°C (○—○) and for 30 min at 37°C (●—●). The hypothalamic cytosol from male rats of the age of 29 days (0.2 ml containing 1.08 mg of protein) was incubated with [1,2,4,5,6,7-^{3}H]-5α-dihydrotestosterone in the same concentration for 30 min at 4°C (△--△).

lation with the results of Davies *et al.*[13] who found a 15% increase of total number of binding sites in females in comparison to males. According to our results the difference in binding is much higher, which proves the advantage of using the microcalorimetric flow system technique.

Increase of temperature from 4° to 37°C resulted in a sharp decrease of 65% from the initial value for the molar enthalpy change after binding of E_2 to the cytosol receptor from female hypothalamus. In the male rats under the same conditions the final molar enthalpy change was 80% of the initial value.

The effect of sex difference is much better expressed for E_2 than for 5α-DHT. Ratio of the molar enthalpy changes for E_2 binding to females and males (F:M) was 2.15 and for 5α-DHT this ratio was 1.22.

If we suppose that the liberated heat or molar enthalpy change is a measure for the number of binding sites then the ratio of molar enthalpy change for E_2:5α-DHT is 2:1 in females and 1:1 for males, at 4°C.

If microcalorimetric determinations, giving precisely determined ratios of the degree of E_2 and 5α-DHT binding, are in some connection with the process of synthesis of 5α-DHT in the male and female hypothalamus and the possible aromatization to estrogens as a second step, then this ratio could be an answer for the physiological sex difference. Further experiments with androgen and estrogen treated animals at the early postnatal period are in progress.

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DISCUSSION

Rousseau. In the few systems where the thermodynamics of steroid–receptor interactions have been studied, the standard free energy change calculated from the equilibrium affinity constant is about 10–15 kcal per mol. Moreover, if one calculates the enthalpy change based on Van't Hoff plots one gets about the same figure, namely the contribution of entropy is minimal. Could you comment on the discrepancy between these values and your figure of about 40 kcal per mol?

Kniewald. On Tables 1 and 2 for estradiol and dihydrotestosterone binding to receptors you'll see that we express the enthalpy values in kcal per mol of hormone and usually you have the enthalpy values in kcal per mol of protein. Our experiments were made for the comparison of binding of estradiol and dihydrotestosterone to receptors from hypothalami at different temperatures. We expressed our results this way because the receptors are not in pure form and therefore our results for enthalpy values are calculated in kcal per mol of hormone, which are pure and the molecular weight is known.

Jensen. As a non physical chemist I must say that I am not only impressed but really amazed that you can make this calorimetry so sensitive as to measure these tiny amounts of receptor complex in hypothalamus. With this kind of sensitivity I think it opens a whole new approach to the study of hormone–receptor complex formation.

Kniewald. By using microcalorimetry it is possible to measure the change of 10^{-8} centigrade. The highest sensitivity is about 10^{-10} cal/sec and we measured in our experiments 10^{-8} to 10^{-9} cal/sec involved in reaction by flow microcalorimetry system.

Jensen. I think this is quite impressive. Now if you were to do this with uterus, where you have a much higher concentration of receptor you should have a much greater response, have you done any studies at all on uterus and prostate?

Kniewald. Yes, we followed the formation of complexes between hormones and receptors from rat prostate, and these results are just published in *Endocrinologia experimentalis Bratislava* **10** (1976) 17.

Clark. I agree with Dr. Jensen, I am fascinated by these observations. However, there are some curious points. How do you account for the energy that is involved in the binding to non-specific sites. If you add estradiol to hypothalamic tissue there will be a considerable amount of binding to sites which are not estrogen receptors. Does that not contribute to the heat produced?

Kniewald. As you know well until now isolated pure receptors do not exist and our results are the final picture of the thermodynamic changes which occurred in system during the measuring, and they show the existing energetic changes in the tissue.

Clark. Could you do something very simple though, just make a simple purification such as an ammonium sulphate precipitation and then run it to see if it changes?

Kniewald. Yes, we could. Thank you for the suggestion.

Munck. Do you find that the heat that's generated is dependent on the concentration of estradiol and achieves a maximum at a concentration of estradiol that would saturate the receptors?

Kniewald. Yes, Tables 1 and 2 show the dependence between the observed heat of binding and the concentration of E_2 and 5α-DHT. The amount of hormone was sufficient to saturate completely the receptor solution in used concentrations. When the observed heat of binding W is plotted *vs* the hormone concentration, the straight-lines are indicated, and from the slopes of lines the enthalpy values are calculated. The straight-lines are the saturation curves in a flow type of microcalorimetry, and in the case of the microcalorimetric measurements by a batch type the saturation curves are the same as in the reaction between enzyme and substrate.