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REDUCTION IN [^{3}H]-CORTICOSTERONE BINDING TO CYTOPLASMIC RECEPTORS IN THE BRAIN OF DIABETIC RATS

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SUMMARY

The binding of [1, 2, 6, 7-^{3}H]-corticosterone was studied in brain cytosol from normal and streptozotocin-diabetic male rats. The experiments were performed under conditions of incubation time (4 h), temperature (0–4°C), time after adrenalectomy (6 days) and corticosterone concentrations (1.2×10^{-8} and 1.15×10^{-9} M) previously established for determining binding activity in the brain of normal rats. The binding of [^{3}H]-corticosterone was found invariably lower in cytosol of the brain from diabetic rats, studied under three different conditions: in non-adrenalectomized animals, in adrenalectomized using a non-saturating corticosterone concentration, and in adrenalectomized plus a saturating steroid concentration. These results support previous contentions that the diminished sensitivity to the negative feedback for steroids which is present in diabetics, may be related to a reduction in binding capacity for corticoids in the central nervous system.

INTRODUCTION

Several reports have appeared in the literature establishing that adrenal hyperfunction is commonly present in diabetic animals. These applied to animals rendered diabetic by subtotal pancreatectomy [1], treatment with drugs [2] or in genetically diabetic animals [3]. The nature of this adrenal overactivity has been partly ascribed to a disfunction of the structures in the central nervous system which regulate the secretion of ACTH [3–6]. The work of L'Age *et al.*[4] in alloxan-treated rats, and our own in streptozotocin diabetic rats [5, 6] concluded that in diabetics the steroid negative feedback elements of the brain do not function properly.

The work of McEwen *et al.* and Grosser *et al.*, have demonstrated that the brain contains macromolecules that bind corticosterone, with all the characteristics of receptors [7, 8]. Whether receptors mediate the negative feedback of steroids in the brain is not entirely proven but it is a likely possibility [9]. Thus, we have determined the binding capacity for [^{3}H]-corticosterone in brain cytosol of normal and diabetic rats, in order to establish whether animals with a diminished sensitivity in the brain to the negative feedback effect, also presented an altered binding activity for corticosterone.

MATERIALS AND METHODS

1. *Experimental animals.* Male Wistar rats of 200–250 g body weight were used. The animals were bilaterally adrenalectomized and used 5–6 days after surgery. During this period they were maintained with rat chow and 0.9% NaCl as drinking fluid *ad libitum*. On the day of the experiment, the rats were anesthetized with ether, bled by cutting the abdominal vessels, and perfused with 60 ml of ice-cold 0.9% NaCl solution through the heart [7]. The brain was taken out from the skull and used for binding studies provided that no blood remained on the adhering membranes. After sectioning the cerebellum and brain peduncles, the remainder of the brain was weighed.

2. *Diabetes induction.* Diabetes was induced by the i.v. administration of 100 mg/kg of streptozotocin, prepared in 0.9% NaCl acidified with 0.05 M citric acid to pH 4.5, according to Junod *et al.*[10]. The animals were periodically checked for urinary glucose by means of Diastix strips (Ames), which usually measured more than 2%. Blood glucose was determined by the method of Somogyi[11].

3. *Corticosterone receptors.* The following procedure was adapted from the work of McEwen *et al.*[7], Grosser *et al.*[8] and Lassman and Mulrow[12]. The brain was homogenized in 5 ml of buffer Tris 10 mM, EDTA 1.5 mM pH 7.0 per gram of tissue and centrifuged at 105,000 *g* for 60 min at 0–4°C, in a Spinco Model L refrigerated ultracentrifuge. The supernatant, referred to as the cytosol fraction, was decanted and used for receptor studies. In a typical experiment, 0.5 ml aliquots of the cytosol were incubated in triplicate with a known amount of [1, 2, 6, 7-^{3}H]-corticosterone, in the presence or absence of a 1000-fold excess of non-radioactive corticosterone. The tubes were incubated for 4 h at 0–4°C with constant shaking, at the end of which period the bound steroid was separated from the unbound fraction by a dextran-coated charcoal method,

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adapted from the work of Baxter and Tomkins[13]. Fifty μl of a suspension in buffer of 1% dextran-10% charcoal was added to the cytosol, the mixture agitated in a Vortex for 30 s and centrifuged at 3000 *g* for 15 min. The radioactivity remaining in the supernatant when incubations were performed without the excess corticosterone was taken to represent the total bound fraction; that present when incubations were additioned of 1000 × corticosterone were considered the non-specifically bound fraction. The difference between these two constituted the [^{3}H]-corticosterone specifically bound to the receptor.

Radioactive counting was performed by transferring an aliquot of the supernatant (0.2 ml) obtained after dextran-charcoal adsorption and centrifugation, to a minivial containing 3 ml of scintillation solution. The radioactivity was measured in a Packard Tri-Carb Model 574 liquid scintillation spectrometer, with a 50% efficiency for tritium. Results were expressed as fmol corticosterone bound/mg protein.

Proteins were determined in the cytosol as follows: an aliquot (0.2 ml) was treated with an equal volume of 10% TCA; after centrifugation, the protein precipitate was resuspended in 0.2 ml of 1 N NaOH. Aliquots of this extract were used to measure proteins by the procedure of Lowry *et al.*[14], using bovine serum albumin as standard.

4. *Materials.* [1, 2, 6, 7-^{3}H]-corticosterone (S.A. 92 Ci/mmol) and Omnifluor were purchased from New England Nuclear (MA, U.S.A.). The counting solution was prepared by addition of 4 g of the scintillator to 1 liter of toluene. [1, 2-^{3}H]-aldosterone (S.A. 53 Ci/mmol) was obtained from Amersham-Searle (IL, U.S.A.). Non-radioactive corticosterone was purchased from Sigma (St. Louis, U.S.A.). Streptozotocin was obtained from Upjohn Laboratories through the kindness of Dr. Raúl Houssay. Dextran T 70 was obtained from Pharmacia (Uppsala, Sweden), and activated charcoal (Norit A) from Amend (New York, U.S.A.). All other chemicals employed were reagent grade.

RESULTS

Figure 1 shows the binding of [^{3}H]-corticosterone to brain cytosol as a function of the incubation time, and using two different steroid concentrations (1.26×10^{-8} M and 1.15×10^{-9} M). Maximal binding was obtained at 4 h of incubation, and it was reduced by 20 h. It was considered that equilibrium was reached by 4 h and consequently this incubation period was employed in routine experiments. Binding was also a linear function of cytosolic protein concentration added to the incubation tubes, in the range of 0.4–3.2 mg/ml aliquot (data not shown).

The specifity of the cytosolic receptor was explored by competition experiments with various natural compounds, used at 25, 50, 100 and 1000-fold molar excess of the concentration of [^{3}H]-corticosterone added (1.15×10^{-9} M). The results in Fig. 2 showed

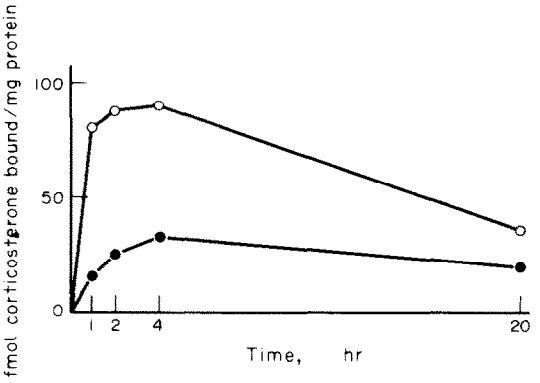

Fig. 1. Time course study of [^{3}H]-corticosterone binding to cytosolic receptors in the brain of adrenalectomized rats. Aliquots of cytosol were incubated at 0–4°C with 1.15×10^{-9} M (lower curve) or 1.26×10^{-8} M (upper curve) [^{3}H]-corticosterone. At the specified times, the bound hormone was separated from the free by the dextran-charcoal method [13]. Results are expressed as fmol/mg protein, and each data point is the average of 2–3 experiments.

that the receptor presented a relative specificity for corticosterone, since both deoxycorticosterone (DOC) and progesterone were as good as competitors as the former compound; aldosterone was less potent and the sex steroids were very poor inhibitors of binding. The order of competitive inhibition was corticosterone = progesterone = DOC > aldosterone > testosterone > 17β-estradiol.

Binding of [^{3}H]-corticosterone to brain receptors was maximal at 0–4°C and it was markedly reduced by incubation at 20 or 37°C (Fig. 3), which suggested that we were measuring an intracellular receptor and not a contaminating transcortin from plasma [7, 8, 15]. Further studies were conducted to differentiate between brain receptors and transcortin. Table 1 shows that brain cytosol binding of corticosterone-[^{3}H] (1.2×10^{-8} M) was considerably decreased by high temperature and by incubation with the SH-blocking agents p-chloromercuribenzoate (PCMB, 1 mM) and *n*-ethyl maleimide (NEM, 2 mM), whereas serum binding was not affected at all by these treatments.

Brain cytosol bound aldosterone-[^{3}H] whereas this compound was not bound to serum (Table 1). Finally, the binding of corticosterone was not distributed uniformly throughout the brain, with the highest binding detected in hippocampus followed by cortex and hypothalamus (data not shown). Thus, we conclude that the brain contains macromolecules with binding properties which differentiate them from the plasma proteins.

Binding of [^{3}H]-corticosterone as a function of the free steroid concentration is shown in Fig. 4. For this purpose, varying amounts of corticosterone (0.2–3.2 ng) were added to the cytosol. A typical hyperbolic curve was obtained, which indicated that saturation was achieved at a [^{3}H]-corticosterone concentration of $\sim 1.2 \times 10^{-8}$ M. When these data were

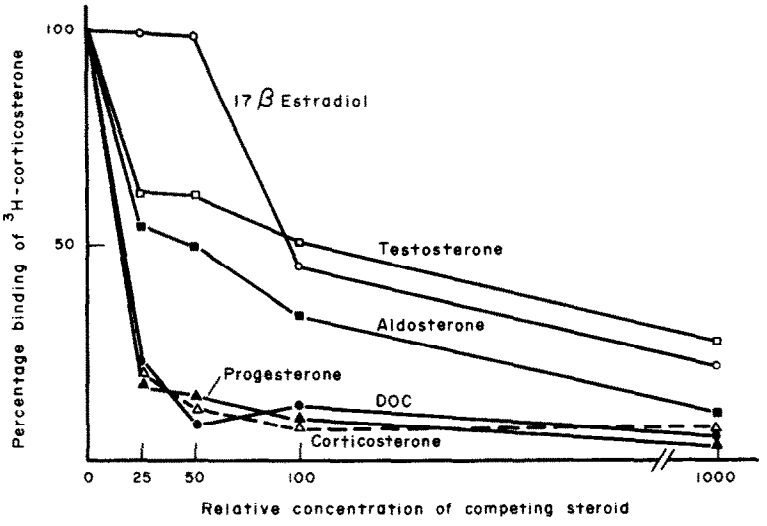

Fig. 2. Inhibition of [^{3}H]-corticosterone binding to cytosolic receptors in rat brain. The cytosol was incubated for 4 h at 0–4°C with 1.15×10^{-9} M [^{3}H]-corticosterone alone or with a 25, 50, 100 or 1000-fold higher concentration of competing steroids. The binding in the absence of competitors was taken as 100%.

plotted according to Scatchard (Fig. 5), a straight line was obtained, indicating a single class of binding sites. Maximal number of binding sites (n) were calculated in three different experiments to be 293 ± 35 (mean ± S.E.) fmol/mg protein, and the association constants (K_a) $3.6 \pm 0.4 \times 10^{-8}\,M^{-1}$, respectively. These values are in the range of those found in the literature [7, 16].

In order to assess the influence of the time after adrenalectomy on [^{3}H]-corticosterone binding rats were divided into four groups: one group was left intact and the other three were adrenalectomized and sacrificed 2, 6 or 10 (or more) days after the operation. Figure 6 shows that intact rats displayed low binding capacity, but this substantially increased at 2 days and reached a maximum at 6 days after adrenalectomy, with a posterior decline to binding levels seen in intact animals. Thus, the 6 day adrenalectomized rat seemed the appropriate preparation to be used in steroid binding studies to brain receptors.

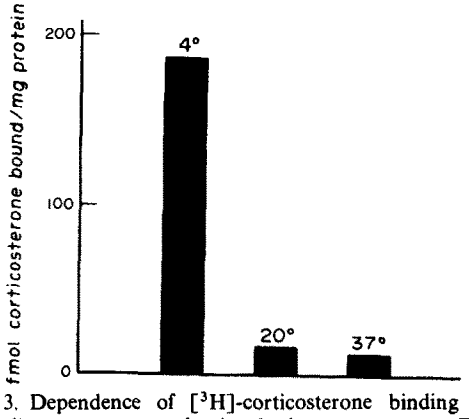

Fig. 3. Dependence of [^{3}H]-corticosterone binding to cytosolic receptors on the incubation temperature. The steroid concentration used was 1.26×10^{-8} M. Other conditions as in Fig. 1.

After establishing the most suitable conditions for studying corticosterone binding, our efforts were concentrated to measure binding in normal and diabetic rats. Three different experiments were dedicated to this purpose, using brain of normal rats or of diabetics sacrificed one month after treatment with streptozotocin (Table 2). Blood glucose in these rats measured up to 500 mg% (5) and all exhibited a pronounced glycosuria (>2%). In the first experiment, intact (i.e., non-adrenalectomized) control and diabetic rats were used, and cytosol was incubated with a low [^{3}H]-corticosterone concentration (1.15×10^{-9} M). In this case, low binding levels were observed, indicating the occupancy of the receptors by the rat's endogenous steroids; however, there was a significantly lower binding in the diabetic group ($P < 0.001$). The second and third experiments were

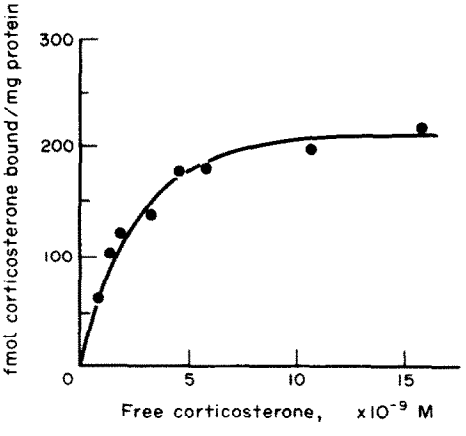

Fig. 4. Relation of specific binding of [^{3}H]-corticosterone in cytosol from adrenalectomized rats to the steroid concentration in the media. The receptor protein is saturated at a steroid concentration of about 1.26×10^{-8} M. Incubations were performed at 0–4°C during 4 h.

Table 1. Steroid binding to brain cytosol and serum proteins under different experimental conditions

Binder	Incubation condition	Steroid binding (fmol/mg prot.) Brain cytosol	Serum (1/25 dilution)
[^{3}H]-Corticosterone	0°C	173.4 ± 12.4	749.4 ± 6.2
[^{3}H]-Corticosterone	20°C	13.1 ± 1.2	779.9 ± 25.1
[^{3}H]-Corticosterone	0°C plus 2 mM NEM	14.3 ± 2.5	811.2 ± 14.5
[^{3}H]-Corticosterone	0°C plus 1 mM PCMB	20.5 ± 2.7	728.2 ± 6.8
[^{3}H]-Aldosterone	0°C	46.0 ± 2.5	0

Brain cytosol and serum from adrenalectomized rats were adjusted to similar protein concentrations and were incubated with 1.2×10^{-8} M [^{3}H]-corticosterone or [^{3}H]-aldosterone.

Data expressed as mean ± S.E. of triplicate incubations. NEM: *n*-ethyl maleimide; PCMB: *p*-chloromercuribenzoate.

carried out with control or diabetic rats sacrificed 6 days after bilateral adrenalectomy. Both a non-saturating (1.15×10^{-9} M, second experiment) and a saturating (1.26×10^{-8} M, third experimental) concentration of corticosterone was tested to bind to cytosols containing identical amounts of protein. In both conditions, the diabetic rats showed lower binding of [^{3}H]-corticosterone (Table 2), which was significantly different than their respective control groups: $P < 0.05$ (second experiment) and $P < 0.01$ (third experiment).

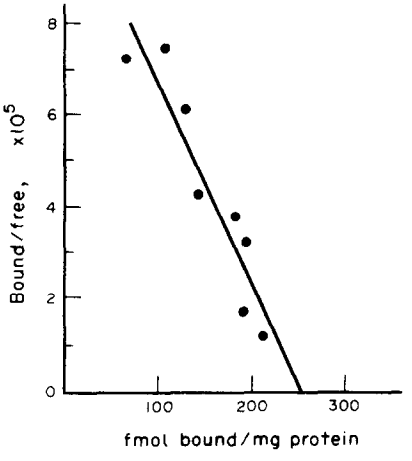

Fig. 5. Scatchard plot analysis of the data presented in Fig. 4. For this experiment, maximal number of binding sites was 254 fmol/mg protein, and K_a 4.3×10^8 M^{-1} respectively.

DISCUSSION

The function of the nervous system-hypothalamic-pituitary-adrenal axis is regulated by several factors, among which the steroids reaching the central structures seem to play an important role. Feedback effects for corticoids have been reported to locate at the hypophysis and hypothalamus [17] but still other sensitive areas have been implicated [18]. Physiologically, an increase in the activity of this axis is counterbalanced by a corresponding increase in adrenal

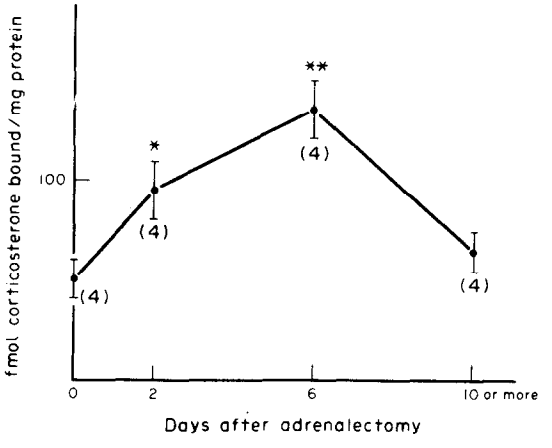

Fig. 6. Binding of [^{3}H]-corticosterone to cytosol receptors in the brain of intact or adrenalectomized rats at different times after operation. Conditions were: corticosterone concentration 1.26×10^{-8} M; 0–4°C and a 4 h incubation time. Data expressed as mean ± S.E.; the figures in brackets represent number of animals studied. *$P < 0.05$; **$P < 0.005$, versus intact rats (0 day of adrenalectomy).

Table 2. Binding of [^{3}H]-corticosterone by brain cytosol of normal and diabetic rats

Group	n	Experimental condition	[^{3}H]-corticosterone added to cytosol	Corticosterone bound (fmol/mg protein)
Control	5	Intact	1.15×10^{-9} M	5.9 ± 0.3
Diabetic	5	Intact	1.15×10^{-9} M	2.4 ± 0.4‡
Control	6	ADX	1.15×10^{-9} M	54.5 ± 5.4
Diabetic	6	ADX	1.15×10^{-9} M	35.4 ± 5.6*
Control	8	ADX	1.26×10^{-8} M	137.0 ± 10.5
Diabetic	9	ADX	1.26×10^{-8} M	93.6 ± 9.7†

Data expressed as mean ± S.E.; n: number of animals. ADX: adrenalectomized.
* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$, versus control animals.

steroid output, which inhibit the secretion of CRH and/or ACTH by acting at one or more of the mentioned central structures.

When pathological conditions prevail, as in chronic stress, the equilibrium is broken and both an increase in the activity of the nervous system-hypothalamic-pituitary-adrenal axis, and of adrenal corticoids, coexist simultaneously [19]. Thus, in stress the steroid negative feedback elements are no longer responding to the influence of circulating corticoids.

In experimental diabetes, it was shown that the deranged metabolism of the diabetic rat acts as a chronic stress [5, 6, 20, 21]. Changes in steroid feedback responses in these diabetic rats and in obese hyperglycemic mice were described [3, 4, 6]. Considering the hypothesis that the "negative feedback mechanism of glucocorticoids operates through a hormonal receptor protein similar to the initial effects of the hormone in other target organs" [9], we measured the binding capacity of the brain for corticosterone in diabetic animals. We first studied some kinetic parameters in normal brain and the following conditions were considered suitable for the study: (a) a 4 h incubation rendered the maximum binding; (b) saturation kinetics were achieved with a corticosterone concentration of $1–2 \times 10^{-8}$ M; (c) the brain macromolecules prepared under our conditions presented binding characteristics which clearly differentiate them from serum proteins; (d) maximal number of binding sites and association constants obtained from Scatchard plot analysis gave values which were in the range of those in the literature [7, 9, 16]; (e) highest binding was observed in rats sacrificed 6 days after adrenalectomy, and (f) competitive binding by other steroids demonstrated that the receptor bound equally well corticosterone, DOC and progesterone. This was not surprising, since DOC and corticosterone may share the same binding protein in the brain [12] and progesterone was also shown to be an effective competitor [7].

The results of Table 2 demonstrated that diabetic brain contains receptors for corticosterone but that there was a reduction of corticosterone binding which amounted to 41–68% of normal, depending on the experimental conditions. Diabetic rats showed lower than normal binding under conditions of receptor occupancy by endogenous steroids or after adrenalectomy; in this case, the lower binding was evidenced using either a non-saturating or a saturating steroid concentration. The reduction in corticosterone binding in diabetics may also apply to other compounds, since Denari and Rosner [22] showed that pituitary and hypothalamus of male and female alloxanized-diabetic rats took up less radioactive estradiol and testosterone than the controls.

Our results suggested that in diabetic rats (and possibly in other stress conditions as well) the activity of neurons that inhibit CRH or ACTH secretion is decreased, due to their lower capacity to bind corticosterone. The disfunction of the nervous system in diabetes probably originated the adrenal overproduction of corticoids, which contributed to the impairment of the disease, due to the marked effects of corticoids on carbohydrate, protein and fat metabolism. This situation in diabetics bears a relationship with that occurring in the testicular feminization syndrome (*Tfm*) rats, in which lack of androgen receptors in the brain conduces to high circulating levels of LH and FSH [23].

Further experiments are planned in our laboratory to study the binding of corticoids in selective brain areas known to concentrate corticosterone, and to complete the physicochemical and kinetic characteristics of the receptor for corticosterone in diabetic rats.

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