ISOLATION AND PRELIMINARY CHARACTERIZATION OF CORTICOSTERONE-RECEPTOR COMPLEXES IN MOUSE PLACENTAL TISSUE

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SUMMARY: The in vitro binding of several radioactive steroids was examined in mouse placental tissue, using Sephadex chromatography to separate the labelled steroid complex from free steroid. Binding was exhibited by cortisol, corticosterone and progesterone, but not cortisone. Cortexolone, an antiglucocorticoid, was tested in the system and found to reduce the binding of the active steroids. The displacement of labelled corticosterone by addition of unlabelled steroids was also examined. Preliminary characterization of the corticosterone receptor using hydrolytic enzymes suggested a protein nature on the basis of degradation by pronase but not by nucleases.

INTRODUCTION: The concept that the first step in steroid hormone action involves specific interaction with receptor molecules is well established (1). Intracellular binding proteins have been demonstrated for estrogens (2), androgens (3) and progesterone (4) as well as the corticosteroids (5,6) in cells of their respective target tissues. Evidence for specific binding of glucocorticoids has been presented for the thymus (7-9) as well as for lymphosarcomas (10,11) and fibroblasts (12). This report presents evidence for the isolation and preliminary identification in mouse placental tissue of intracellular protein component(s) which specifically bind corticosterone.

ABBREVIATIONS: KEP, Krebs-Eggleston phosphate buffer; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid tetrasodium salt.

MATERIALS AND METHODS: Cortisol-4-^{14}C (52 mCi/mmole), cortisone-1,2-^{3}H (51.6 Ci/mmole) and progesterone-4-^{14}C (52.8 mCi/mmole) were purchased from New England Nuclear Corp.; corticosterone-

1,2-^{3}H (36 Ci/mmole) from Amersham/Searle. Non-radioactive cortexolone was obtained from Nutritional Biochemical Corp.; 11-epicortisol from Mann Research Labs; and cortisol, cortisone, progesterone, and corticosterone from Sigma Chemical Co. Pronase (45,000 PUK units /mg), deoxyribonuclease (42,300 Dornase units/mg) and ribonuclease (47 Kunitz units/mg) were all obtained from Calbiochem.

Experiments were performed on randomly bred female mice of the SWV strain raised at this university. The animals were fed a diet of Purina Breeder Chow and water ad lib. and were used between day 13-18 of pregnancy.

Animals were killed and the placentas removed and placed on ice. Extraneous tissue was removed and the placentas (2/sample) were then lightly homogenized with 2-3 strokes of a loose-fitting tissue grinder in 2 ml KEP buffer pH 7.4 containing 2.8 mM glucose. Radioactive steroid, alone or with non-radioactive steroids were added and the samples left on ice for 15 min with occasional agitation. The samples were then incubated at 37°C for 10 min in a shaking water bath, put back on ice and diluted by addition of 50 ml of cold 1.5 mM $MgCl_2$. This procedure has been used by Munck and co-workers (7) to shatter cell membranes, disrupting cytoplasmic material but leaving nuclei intact (as evidenced by examination under a microscope). After sitting on ice for 10-15 min, each sample was centrifuged at 105,000 g for 5 min in a refrigerated ultracentrifuge and the pellet (intact nuclei) resuspended in 0.7 ml of cold buffer composed of 0.6 M KCl, 10 mM Tris and 1.5 mM EDTA at pH 8.0. The nuclei were then homogenized with 8-10 strokes of a tight-fitting Ten Broeck ground glass homogenizer and recentrifuged at 105,000 g for 5 min. An aliquot (usually 0.5 ml) of the supernatant was then removed

and the bound steroid separated from the free compound by passage through 11 x 180 mm columns of Sephadex G-25 (Pharmacia) using an elution buffer of 0.6 M KCl, 10 mM Tris, 1.5 mM EDTA (pH 8.0). The elutions were carried out at 6°C and the peak of bound material could be collected 10 min after application of the sample. Fractions of approximately 1 ml were collected and the macromolecular peak was identified by protein determinations using the method of Lowry, et al. (13).

Radioactive determinations were made on 0.1 ml aliquots added to 10 ml of scintillation fluid (toluene plus fluors) along with an appropriate amount of Bio Solv solubilizer BBS-3 (Beckman Instrument Co.). The samples were then assayed in a Packard Tri-Carb liquid scintillation spectrometer, Model 3003.

In the experiments with the hydrolytic enzymes a cell-free nuclear supernatant was used to test the effect on specific binding. The nuclei were prepared without addition of steroid or incubation at 37°C. After homogenization and centrifugation the supernatant was divided into aliquots (0.45 ml) and incubated at 37°C for 30 min with ^{3}H-corticosterone and the appropriate enzymes. The samples were then cooled on ice and treated on the Sephadex columns at 6°C as before.

RESULTS AND DISCUSSIONS: All steroid systems studied to date appear to involve specific binding to a protein receptor in their mechanism of action. In a previous paper (14), we reported the observation that corticosteroids inhibited the incorporation of ^{14}C-glucose into fetuses of several strains of mice. Therefore it did not seem unreasonable that the placenta might contain molecules which interact specifically with steroid hormones. When placental homogenates were incubated at 37°C with ^{14}C-

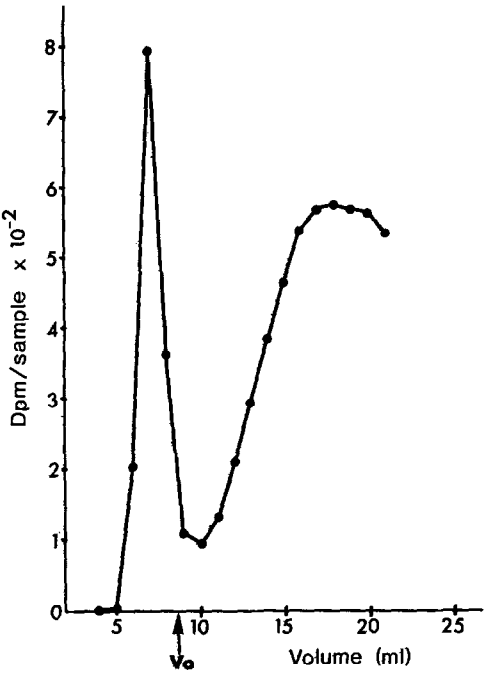

Fig. 1. Isolation of ^{14}C-cortisol-receptor complexes on Sephadex G-25 at 6° C.

Homogenate of placental tissue was incubated with ^{14}C-cortisol (0.96 μM) for 15 min at 0° C, then 10 min at 37° C. The sample was then diluted with 1.5 mM $MgCl_2$, and the nuclear supernatant prepared and eluted through Sephadex G-25 as described in Methods. Radioactivity isolated in the void volume was taken to be associated with the macromolecular peak and used as a measure of specific binding.

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cortisol, two peaks of radioactivity were isolated on Sephadex G-25 (Fig. 1). The intense peak occurred in the void volume and coincided with the protein peak, indicating an association of the radioactive cortisol with a macromolecule. When radioactive corticosterone, cortisone, and progesterone were each examined for binding, corticosterone and progesterone both gave sharp peaks of radioactivity in the same region (volume 6-8 ml) whereas cortisone exhibited only a minor increase in counts in that region. Thus the hormonally-active steroids demonstrated the ability to form complexes in this system while cortisone, a

Table I. Effect of cortexolone on steroid binding in placental homogenates.

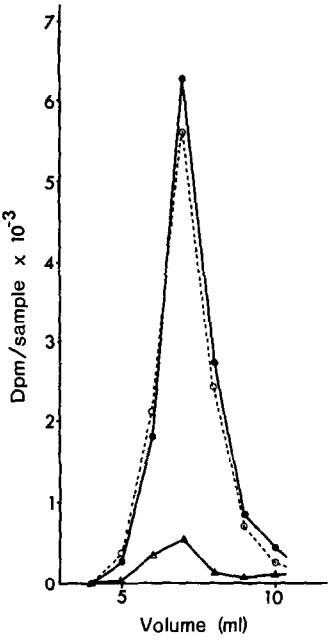
Homogenates of placental tissue were incubated with either ^{14}C-cortisol, ^{3}H-corticosterone, or ^{14}C-progesterone alone or together with cortexolone for 15 min at 0° C, then 10 min at 37° C. The samples were then diluted with 1.5 mM $MgCl_2$, and the nuclear supernatants prepared and eluted through Sephadex G-25 as described in Methods. Radioactivity isolated in the void volume was taken to be associated with the macromolecular peak and used as a measure of specific binding.

STEROID(S) AND CONCENTRATION	SPECIFIC BINDING	% OF CONTROL
^{14}C-Cortisol (0.96 μM)	2,520	
^{14}C-Cortisol (0.96 μM) + 10^{-5}M Cortexolone	920	37
^{3}H-Corticosterone (1.01 μM)	17,100	
^{3}H-Corticosterone (1.01 μM) + 10^{-5} M Cortexolone	5,690	33
^{14}C-Progesterone (0.85 μM)	2,260	
^{14}C-Progesterone (0.85 μM) + 10^{-5} M Cortexolone	1,420	63

steroid which only has *in vivo* glucocorticoid activity (by virtue of its conversion to cortisol) was unable to bind.

Cortexolone, used in the thymus system by Munck (15), Rosen (16) and others as an antiglucocorticoid also exhibited this property with placental binding. Addition to the incubation mixture of 10^{-5} M cortexolone resulted in a lowering of the radioactive peak of specific binding for all three of the hormones tested (Table I). Progesterone binding was affected the least whereas the displacement of corticosterone was almost twice as great, suggesting a greater affinity for corticosterone. Since corticosterone is also the natural glucocorticoid in mouse plasma (17), it was used in further studies.

The specificity of ^{3}H-corticosterone binding was studied by testing the effects of addition of cortisol and its biologically



	TOTAL (tubes 4-10) COUNTS (dpm)	PROTEIN (mg)
^{3}H-corticosterone + 2μM cortisol —△—	1,250	2.06
^{3}H-corticosterone + 2μM epi-cortisol --⊖--	11,500	2.00
^{3}H-corticosterone alone —●—	12,400	2.10

Fig. 2. Effect of cortisol and epi-cortisol on ^{3}H-corticosterone binding in placental homogenates.

Homogenates of placental tissue were incubated with ^{3}H-corticosterone (13.8 nM) alone or together with either 11α-cortisol or 11β-cortisol for 15 min at 0° C, then 10 min at 37° C. The samples were then diluted with 1.5 mM $MgCl_2$, and the nuclear supernatants prepared and eluted through Sephadex G-25 as described in Methods. Radioactivity associated with the protein peak was used as a measure of specific binding.

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inactive epimer, 11-epicortisol. As shown in Figure 2, the active epimer almost abolished the binding completely whereas 11-epicortisol reduced the binding by only 7%. Other steroids were also used to test their effectiveness in displacing ^{3}H-corticosterone (Table II). Progesterone and corticosterone proved to be as effective as 11β-cortisol whereas cortisone was partially effective. It has not been determined whether or not the activity of the cortisone might be due to traces of contaminating steroids or to the activity of the enzyme 11β-hydroxysteroid:NADP oxidoreductase, known to occur in placenta (18). Of the radioactive corticosterone bound, greater than 80% has been recovered

Table II. Competitive interaction of Steroids with ^{3}H-corticosterone binding in placental homogenates.

Homogenates of placental tissue were incubated with ^{3}H-corticosterone (13.8 nM) alone or together with various unlabelled steroids (2 μM) for 15 min at 0° C, then 10 min at 37° C. The samples were then diluted with 1.5 mM $MgCl_2$, and the nuclear supernatant prepared and eluted through Sephadex G-25 as described in Methods. Radioactivity associated with the protein peak was used as a measure of specific binding.

COMPETING STEROID	SPECIFIC ACTIVITY OF BINDING (dpm/mg protein)	% OF CONTROL
None	5120	100
Epi-Cortisol	4990	97
Cortisone	2190	43
Progesterone	820	16
Cortisol	650	13
Corticosterone	510	10

Table III. Effect of hydrolytic enzymes on ^{3}H-corticosterone-receptor complexes.

A nuclear supernatant was prepared from placental tissue as described in Methods. ^{3}H-corticosterone (55 nM) and 50 λ of enzyme was added to 0.45 ml aliquots and incubated for 30 min at 37°C (0.05 ml buffer added to controls). The samples were then cooled to 0°C and eluted through Sephadex G-25 as described in Methods. Radioactivity associated with the protein peak was used as a measure of specific binding.

TREATMENT	SPECIFIC BINDING (dpm)	% OF CONTROL
No Additions	971,600	100
Pronase, 15 mg/ml	93,700	10
Deoxyribonuclease, 10 mg/ml	1,095,700	113
Ribonuclease, 10 mg/ml	1,003,700	103

after TLC in the zone corresponding to unmetabolized corticosterone.

The susceptibility of the ^{3}H-corticosterone complex to the effects of hydrolytic enzymes was examined by treating the supernatant of the nuclear preparation with the labelled steroid and

various enzymes (Table III). Pronase decreased the specific binding by 90% whereas deoxyribonuclease and ribonuclease had no effect. This result is comparable to those of other workers (7, 19,20) leading to a tentative conclusion that this receptor molecule, as with all receptors examined to date, is at least in part protein.

This investigation of steroid binding in placental tissue has uncovered the existence of receptors in a tissue where not only corticoids, but progestins and estrogens play a physiological role. The placenta appears to be a very interesting system and, like the uterus (21), may contain binding proteins for more than one type of steroid. From our data it appears to bind both corticoids and progestins and there also appears to be a binding molecule for estradiol-17β resulting in a complex which sediments at 4s on sucrose density gradient (22). If binding proteins are present for all the hormones which interact with the placenta then the possibility of "cross-affinity" among the receptorsmay occur (21). The possible role of this under physiological conditions could be associated with hormonal control over placental and fetal growth and viability.

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