Journal of Steroid Biochemistry, Vol. 10, pp. 285 to 289
Pergamon Press Ltd. 1979. Printed in Great Britain

SPECIFIC BINDING OF [^{3}H]-16β-HYDROXYDEHYDROEPIANDROSTERONE BY RAT KIDNEY*

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(*Received* 20 *July* 1978)

SUMMARY

16β-Hydroxydehydroepiandrosterone (16β-OH-DHEA) can influence electrolyte excretion in the rat and has been reported to be elevated in the urine of patients with low-renin essential hypertension. This steroid does not bind to the mineralocorticoid receptors in rat or human kidney that bind aldosterone and other sodium-retaining steroids. Thus, actions of 16β-OH-DHEA may be mediated through other binding sites. To obtain a direct indication of how cells interact with 16β-OH-DHEA, we studied the binding of the radioactively labeled steroid. Limited capacity binding of [^{3}H]-16β-OH-DHEA was demonstrated after incubating the steroid with kidney slices at 37°C (apparent equilibrium dissociation constant (K_D approximately 0.3 μM) or cytosol at 0°C (K_D approximately 3 μM). Specific binding activity by kidney cytosol was respectively 6-fold and 40-fold greater than by liver or brain cytosol. Nonradioactive testosterone, progesterone and the spironolacton SC14266 had detectable but weak activities (as compared with non-radioactive 16β-OH-DHEA) for inhibition of [^{3}H]-16β-OH-DHEA. No significant competition was observed with androstenediol, aldosterone, deoxycorticosterone, corticosterone and 16-oxo-androstenediol at concentrations to 10 μM. A heat-dependent nuclear transfer mechanism for 16β-OH-DHEA was not observed.

Thus, limited capacity binding sites exist in rat kidney which can recognize 16β-OH-DHEA. The sites are distinguishable from the previously characterized mineralocorticoid, glucocorticoid and androgen receptors, and differ from known steroid receptors in that they lack an obvious nuclear transfer mechanism. The physiological role of the binding sites detected in these studies is not known.

INTRODUCTION

There has been considerable recent interest in the potential role of steroids other than those classically considered to be major mineralocorticoids as being involved in the pathogenesis of human hypertension, especially that associated with low levels of plasma renin (for references, see [1–9]). The C_{19} steroid, 16β-OH-DHEA is one which has received recent attention. Sennett and coworkers reported this steroid to be elevated in the urine of patients with low renin hypertension [1, 2]. Liddle and Sennett also demonstrated that 16β-OH-DHEA could influence electrolyte excretion in the rat and this effect was blocked by spironolactone [1, 2]. That the steroid affects urinary sodium and potassium under certain restricted experimental conditions was confirmed by Funder and coworkers [3], but not noted by ourselves [4]. Recently, Nowaczynski and coworkers confirmed the finding of elevated urinary 16β-OH-DHEA levels in low-renin hypertensive patients [9]. However, Ulick and Ramirez were unable to detect such elevations with the use of slightly different methodology [10]. More recently, Sennett and coworkers, using a modified urinary chromatographic procedure reported that the "mineralocorticoid" activity detected by bioassay in their 16β-OH-DHEA fraction could not be totally accounted for by 16β-OH-DHEA [11]. In these studies, they did not find an increase in urinary 16β-OH-DHEA levels in patients with low-renin hypertension. Thus, it is not known whether 16β-OH-DHEA (or other steroid precursors or metabolites) is elevated or plays a role in low-renin hypertension. In any event, more information is necessary about this and other steroids that are potential candidates for influencing salt balance. We [8, 12, 13] and others [3] found that 16β-OH-DHEA does not bind to the renal mineralocorticoid receptors that bind aldosterone and other known sodium-retaining steroids [3, 8, 12, 13] nor does it bind the glucocorticoid, androgen or estrogen receptors [3]. Since no information is available about the nature of the interactions of 16β-OH-DHEA with the kidney, we have, in the current studies, investigated the nature of the binding of 16β-OH-DHEA with kidney with the use of tritiated steroid.

* Supported by NIH Grants 5-RO1-HL-16918, RO1-HL-18730-01, and NIH contract 74C-826-CC.
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MATERIALS AND METHODS

The 7α-bromo derivative of 16β-OH-DHEA diacetate was prepared [14], immediately placed in dry ice and sent to New England Nuclear Corporation for catalytic dehalogenation with tritium [15]. This product is unstable and attempts to purify it were unsuccessful, yielding multiple spots by thin layer chromatography after purification by chromatography or crystallization. This experience has been reported by others [15]. The 16β-OH-DHEA diacetate was partially purified from the crude dehalogenated mixture by preparative thin layer chromatography using silica Gel GF-254 (Merck) in the system iso-octane–acetone (90:10, V/V). The plates were scanned in a Packard chromatoscanner, and the peak of radioactivity corresponding to authentic 16β-OH-DHEA diacetate determined in a parallel run after spraying with blue tetrazolium, was scraped and eluted with a mixture of ethyl acetate and water (10:1, V/V). The ethyl acetate was evaporated at 40°C under nitrogen.

The material was spotted on silica gel plates impregnated with silver nitrate (1 g of silica per 2 ml of a 10% solution of silver nitrate was used to coat the plates at a thickness of approximately 500 microns; the plates were allowed to dry in the dark) and developed 3 times in the system toluene:ethyl acetate (97:3, V/V). A partial separation of 16β-OH-DHEA diacetate from 16β-OH-epiandrosterone diacetate was obtained. The area corresponding to 16β-OH-DHEA diacetate was eluted as above and evaporated; 2 ml of phosphate buffer (0.2 M, pH 7.0) containing 10 mg of a malt a-amylase (Sigma) was added. The mixture was kept at room temperature for 48 h before it was extracted with ethyl acetate, washed with water, evaporated under nitrogen, spotted on a silver nitrate-impregnated silica gel plate, and developed three times in the system chloroform–acetone (92:8, V/V) to clearly separate 16β-OH-DHEA from its isomer 16-oxo-androstenediol and from 16α-OH-DHEA. No evidence of isomerization of 16β-OH-DHEA eluted from the last silica gel purification was obtained when checked by mixing an aliquot with authentic 16β-OH-DHEA and 16α-OH-DHEA and 16-oxo-androstenediol and developing in two different chromatographic systems. These systems were: (i) a celite plate (5 × 20 cm) using propylene glycol as the stationary phase while developing 3 times with toluene; and (ii) a silica gel plate (5 × 20 cm) developed three times with toluene:ethanol (93:7, V/V). The above plates were scanned initially and then sprayed with blue tetrazolium to localize the cold steroids. (Both systems accomplish excellent separation of the isomers.) Over 95% purity was obtained by this method. The specific activity determined by a radioimmunoassay similar to that described elsewhere [10] was 4–4.5 Ci/mmol.

Nonradioactive steroids. Nonradioactive steroids were obtained from Sigma Chemical Company.

Binding studies. Male Sprague–Dawley rats (160–200 gm) were adrenalectomized and maintained on 1% saline for 4 days to 3 weeks prior to use. Rats were sacrificed by cervical dislocation; the kidneys perfused with ice-cold PBS (0.025 M KH_2PO_4, 0.09 M NaCl, pH 7.4), decapsulated and placed in ice-cold PBS prior to further processing.

For cytosol binding, the kidneys were minced and then homogenized in 1 volume (v/w) of buffer A (2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM N-tris (hydroxymethyl) methyl glycine (tricine), 3.0 mM dithiothreitol and 5% glycerol, pH 7.4). The homogenate was centrifuged at 120,000 *g* for 90 min at 0°C. The supernatant cytosol was removed and used in incubations at an additional 1:3 dilution with the same buffer. The final protein concentration in incubations was approximately 4 mg/ml. Bound steroid was assayed by a charcoal technique described elsewhere [16].

For tissue slice studies, the kidneys were halved, chopped bi-directionally and resuspended in PBS as described previously [12], and 0.3 ml aliquots were added to incubation vials containing 1.8 ml of incubation solution B (133 mM NaCl, 0.5 mM $MgCl_2$, 5 mM Tris–HCl and 5 mM glucose, pH 7.4). For competition studies the incubation vials contained [^{3}H]-16β-OH-DHEA (final at 0.9 μM) and various amounts of competitor steroids at 30 mM to 10 μM. Following 30 min incubations at 37°C, the tissue was pelleted by centrifugation (600 *g*, 5 min), washed 3 times with ice-cold PBS and resuspended in 0.2 ml buffer A. Each sample was homogenized in a glass tube with a Teflon pestle (6 strokes at 2000 rev./min) and centrifuged at 9000 *g*. Cytosol macromolecular bound radioactivity was separated from free hormone by Sephadex G-25 (Pharmacia) column (0.4 cm × 9.5 cm) chromatography as described elsewhere [8] and assayed by counting (at 45% efficiency) in 5 ml of 4 gm/liter Omnifluor (New England Nuclear) in toluene with 25% Triton-X-100. Binding in each sample was normalized to the protein concentration determined by the method of Lowry[17].

In all cases, "background" non-specific binding was determined by parallel incubations which contained (in addition to [^{3}H]-16β-OH-DHEA) a 1000-fold excess of nonradioactive 16β-OH-DHEA. With cytosol and slices respectively, background binding of [^{3}H]-16β-OH-DHEA was 10% and 20% of the total binding. In all cases, this background binding was subtracted from the total binding to yield specific binding [16].

For determination of authenticity of macromolecular-bound radioactivity, tissue slices were incubated with radioactive hormone (37°C), homogenized and gel filtered as above. The macromolecular-bound fraction eluted from the gel was extracted twice with methylene chloride (10 volumes) which was subsequently evaporated under nitrogen. Thin-layer chromatography was accomplished by spotting on silica gel plates (FG 254, Merck) and developing in the system toluene–ethanol (92:8, V/V). The plate was analyzed in 1 cm sections for radioactivity.

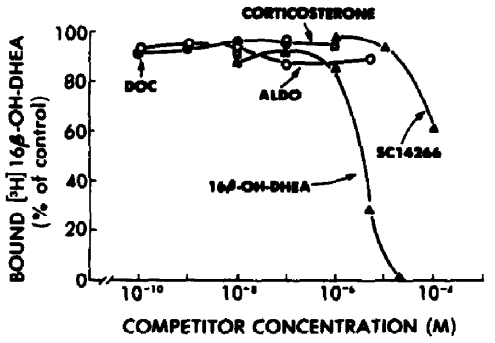

Fig. 1. Competition by nonradioactive deoxycorticosterone (DOC [●]), aldosterone (○), corticosterone (□), spironolactone (SC14266 [△]) and 16β-OH-DHEA (▲) for [^{3}H]-16β-OH-DHEA (0.1 μM) binding by rat kidney cytosol at 0–4°C. The control (100%) represents specific binding in the absence of any competitor and was 40,000 c.p.m./sample. All points represent duplicate determinations. Not shown, nonradioactive 16-oxo-androstenediol (at 10 and 50 μM) did not inhibit binding.

RESULTS

Specific binding of [^{3}H]-16β-OH-DHEA by kidney cytosol

The specificity of [^{3}H]-16β-OH-DHEA binding is shown in Fig. 1, in which [^{3}H]-16β-OH-DHEA was incubated in cytosol at 0°C with various concentrations of several steroids. As indicated, binding was inhibited by non-radioactive 16β-OH-DHEA and (to a lesser extent) by the spironolactone SC 14266. In contrast, no competitive inhibition by aldosterone, deoxycorticosterone or corticosterone was observed at concentrations at which these steroids would clearly competitively inhibit their binding by their own receptors. Since corticosterone and deoxycorticosterone were not examined at concentrations above 1 μM (a concentration required for minimal competitive inhibition of [^{3}H]-16β-OH-DHEA binding by 16β-OH-DHEA itself) the possibility that these steroids can bind to the 16β-OH-DHEA sites with an affinity comparable to 16β-OH-DHEA cannot be excluded. It is clear, however, from these data, that these 16β-OH-DHEA-binding sites exhibit a specificity of steroid binding quantitatively different from the known classes of steroid receptors. The 16-oxo isomer (16-oxo-androstenediol) of 16β-OH-DHEA was also examined for competitor activity and did not inhibit binding at concentrations to 50 μM (data not shown).

Specific binding of [^{3}H]-16β-OH-DHEA was also demonstrated after the hormone was incubated with kidney slices at 37°C. The concentration dependence of binding is shown in Fig. 2 in the form of a Scatchard analysis of the data [17]. The roughly linear relationship is consistent with (but does not demonstrate) a single class of sites on a thermodynamic basis. Under these conditions, the apparent equilibrium dissociation constant, K_D, is about 0.3 μM and the binding capacity is at least 20 pmol/mg protein. The specificity for binding in terms of steroid structure in the case of the incubations with kidney slices is demonstrated in Fig. 3. As shown, the binding is readily inhibited by nonradioactive 16β-OH-DHEA, but testosterone and progesterone are only mildly effective as competitors, even at concentrations which begin to approach the solubility of steroids.

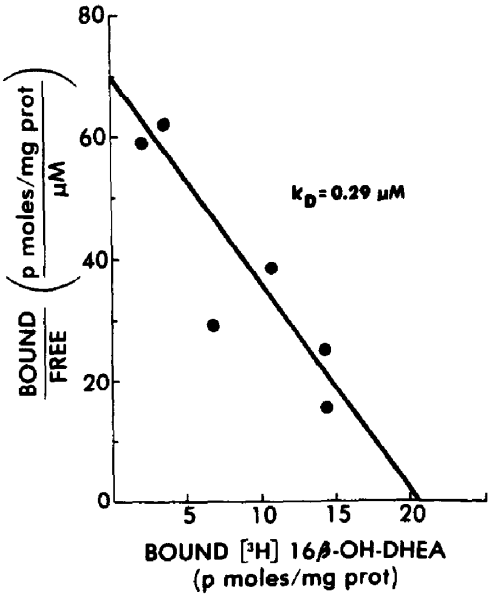

Fig. 2. Scatchard[18] analysis of specific binding of various concentrations of [^{3}H]-16β-OH-DHEA by rat kidney tissue slices at 37°C incubated as described in Methods. Free steroid concentration was determined by measuring the [^{3}H]-16β-OH-DHEA in the supernatant medium at the end of each incubation. All points represent single determinations.

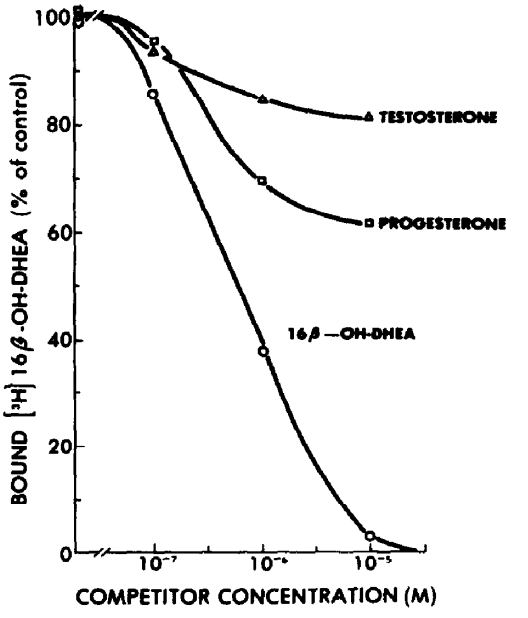

Fig. 3. Competition by nonradioactive testosterone (△), progesterone (□), and 16β-OH-DHEA (○) for [^{3}H]-16β-OH-DHEA (0.9 μM) binding by rat kidney tissue slices at 37°C. The control (100%) represents specific binding in the absence of competitor and was 12,500 c.p.m./mg protein. All points represent duplicate determinations.

Table 1. Effect of cytosol and temperature on nuclear binding of [^{3}H]-16β-OH-DHEA*

Incubation conditions	Temperature	c.p.m. specifically bound per sample in: Cytosol		Nuclei	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2
Cytosol alone	0°	4880	9600	—	—
	22°	24,000	12,500	—	—
Cytosol plus nuclei	0°	15,500	9900	7100	300
	22°	29,500	10,300	6700	150
Nuclei alone	0°	—	—	—	1230
	22°	—	—	—	3570

* Cytosol was prepared as described in Methods and incubated for 6 h at 0°C with [^{3}H]-16β-OH-DHEA (0.1–0.3 μM). Specific binding was measured as described (Methods). Nuclei were prepared as previously described (20, 21) and aliquots (0.5 ml) were added to 0.5 ml of buffer A or previously bound cytosol and incubated for 25 min at either 0° or 22°C. Specific nuclear binding was determined by washing the nuclei 3 times with cold PBS and assaying for radioactivity (Methods).

Tissue distribution of 16β-OH-DHEA binding sites

To obtain some indication of the distribution of binding sites for 16β-OH-DHEA, we investigated two other tissues, liver and brain. Specific binding was demonstrated in the latter tissues, but was lower than that demonstrated in kidney such that renal binding activity of specifically-bound steroid (66 pmol/mg protein when 0.3 μM [^{3}H]-16β-OH-DHEA was incubated with cytosol) was 6-fold higher than that in liver (10 pmol/mg protein) and 40-fold higher than that in brain (1.6 pmol/mg protein). The amount of binding of [^{3}H]-16β-OH-DHEA in pmoles/mg protein greatly exceeds that of [^{3}H]-aldosterone and [^{3}H]-dexamethasone determined in previous studies (i.e., 0.01–2 pmol/mg protein) [16, 19].

The question of nuclear transfer of 16β-OH-DHEA

Since many physiological actions of steroid hormones are mediated through the hormonal binding to cytoplasmic receptors and subsequent steroid-receptor complex transfer to the nucleus, it was of interest to know whether the binding sites for 16β-OH-DHEA behave in an analogous fashion. Experiments testing nuclear transfer were performed at conditions identical to those in which nuclear transfer of other classes of steroid receptors is achieved [20, 21]. There was specific nuclear binding of [^{3}H]-16β-OH-DHEA (Table 1); however, this was not stimulated by increasing the temperature and in fact was lower at 22°C than at 0°C. Further, specific binding was observed when nuclei were incubated with the [^{3}H]-steroid in the absence of cytosol (Expt. 2) and exceeded the binding observed in the presence of cytosol.

Thin layer chromatography analysis of the macromolecular-bound steroid

Renal tissue slices were incubated with [^{3}H]-16β-OH-DHEA and macromolecular bound radioactivity was extracted as described in the methods, and was analyzed by thin layer chromatography. The area of the chromatogram corresponding to authentic 16β-OH-DHEA standards run in parallel represented greater than 50% of the spotted radioactivity. A significant amount of radioactivity (~30%) migrated with 16-oxo-androstenediol (the isomer of 16β-OH-DHEA). Whether this isomerization occurred during the incubation of [^{3}H]-steroid with the tissue or during subsequent extraction procedures is unclear because of the known instability of these steroids in terms of isomerization [11]. In addition, a small amount (~10%) of an apparently more polar compound was detected, but this was not identified.

DISCUSSION

In the current studies, limited capacity renal binding sites for 16β-OH-DHEA were demonstrated. These sites were also specific for the kidney in the sense that binding of radioactive 16β-OH-DHEA by kidney exceeded that of either liver or brain cytosol (by 6- and 40-fold, respectively). The binding sites differ from the receptors for androgens, progestins, glucocorticoids and mineralocorticoids as these steroids showed little or no ability to displace radioactive 16β-OH-DHEA. The sites are also different from the other steroid receptors [16, 19] in their concentration. Binding to 60 pmol/mg protein was observed, and this exceeds (by 50-100-fold) the binding capacity of other classes of steroid hormone receptors detected in the same tissue or in other tissues such as liver. Some specific binding of 16β-OH-DHEA was also observed when the steroid was incubated with isolated nuclei. However, the 16β-OH-DHEA binding sites detected differ from those characteristic of other steroid receptors in that a detectable temperature-dependent, cytosol-stimulated nuclear transfer mechanism was not apparent.

Because of the stability of the 7α-bromo derivative of 16β-OH-DHEA, and the multiple steps required to isolate [^{3}H]-16β-OH-DHEA, it might be questioned whether the binding of radioactivity was actually due to [^{3}H]-16β-OH-DHEA rather than some contaminant. We feel that this is unlikely for three reasons. First, care was exercised (see Methods) during the isolation

of the original steroid to identify and purify 16β-OH-DHEA. Secondly, analysis of the tissue-associated radioactivity after extraction and thin layer chromatography suggested that the bound material was authentic 16β-OH-DHEA. Finally, the calculated binding affinity was similar when examined by either competition by nonradioactive 16β-OH-DHEA for [^{3}H]-16β-OH-DHEA binding or by Scatchard plots of the binding of the radioactively labelled compound.

The finding of renal sites specific for 16β-OH-DHEA does raise the question of whether there could be biologically important receptors for this steroid. This question cannot be answered from the current findings. The finding that these binding species differ from the known steroid hormone receptors in terms of binding capacity and lack of an obvious nuclear transfer may reflect the fact that the sites are not receptors. However, if there are actions of this or other steroid metabolites independent of the major steroid classes, it does not follow *a priori* that the receptor system or the mechanisms would be identical. It is equally plausible that these sites reflect a metabolizing enzyme, a transport protein or a protein with some other function. What is clear from these current investigations of [^{3}H]-16β-OH-DHEA binding is that a cellular recognition function has evolved that can distinguish 16β-OH-DHEA from other classes of steroids. Therefore, 16β-OH-DHEA could have a receptor system independent of the traditional classes of steroids.

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