Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 22-29

PHYSICOCHEMICAL CHARACTERIZATION OF [^{3}H] DHEA BINDING IN RAT LIVER

Mohammed Kalimi* and William Regelson

Departments of Physiology, Microbiology and Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298

Received August 25, 1988

SUMMARY: Dehydroepiandrosterone (DHEA), the native clinical steroid and steroid precursor, may have a targeted physiologic role. A high affinity (Kd 2.3nM) and steroid specific [^{3}H] DHEA binding macromolecule in male Sprague-Dawley rat hepatic cytosol suggests that DHEA may have receptor mediated physiologic action. ^{3}[H] DHEA binding was highest in the liver followed by kidney and testis cytosols. Sulfhydryl reagents such as N-ethylmalemide and iodoacetamide inhibited the binding of [^{3}H]DHEA by up to 60-70%. The DHEA-macromolecular complex was stable at 35°C. and addition of 5mM molybdate or 0.3M KCl increased stability. Interestingly, rat liver cytosol, specific binding at 4° C increased by almost 40-50% with addition of 0.1M NaSCN or 0.3M KCl. Sucrose gradient analyses showed a 7-8 S macromolecular complex in the low salt and 3-4 S complex under high salt conditions. The [^{3}H] DHEA- macromolecular complex shows minimal temperature dependent activation in vitro at 25°C as judged by binding to DNA-cellulose. The results suggest a specific high affinity macromolecule for DHEA in rat liver cytosol with unique physicochemical properties.

INTRODUCTION: Dehydroepiandrosterone (DHEA) is a major clinical cortical steroid which declines with chronologic age (1). For many years, DHEA has been thought to be important only as an intermediate source for the sex steroids. That DHEA may have alternative roles of primary importance is seen in its wide range of biological activity including anticarcinogenic action (2), anti-obesity (3), anti-diabetic effects (4) and CNS effects (5). Although DHEA is now seen to have many biochemical, physiological and immunological effects in rodents, (6,7) our knowledge regarding how DHEA exerts its broad modulating effects remains very limited. Hardly any information is available regarding DHEA's binding activity in various target tissues and since DHEA is a steroid, it is likely that, like other steroid

*To whom all correspondence should be addressed.

0006-291X/88 $1.50

hormones, DHEA responses may be mediated by specific high affinity receptors present in the subcellular fractions of target tissues such as cytosol and/or nuclei. This is particularly pertinent to recent data suggesting an affinity of DHEA for rat CNS binding (5). It is possible that there are distinct binding sites for DHEA in target cells which mediate observed physiologic responses. Therefore, the identification and characterization of DHEA binding in target cells is of considerable scientific and potential clinical interest.

Alternatively, DHEA might cross react with other steroid hormone receptors to modulate their activity. In this regard, there is one report suggesting that there is competition by DHEA with the receptor binding of estradiol and estrone in post menopuasal females (8). On the other hand. Poortman et al. (9) detected no DHEA or DHEA sulfate binding activity to estrogen receptors. In this paper, we describe DHEA binding, in vitro activation, and the physicochemical properties of a DHEA high affinity protein in rat hepatic cytosol. The results presented here will help us understand the molecular mechanism by which DHEA may mediate broad biochemical responses during cellular growth, differentiation, transformation and senescence.

MATERIALS AND METHODS: Male Sprague-Dawley rats (200-250 g) were obtained from Charles River Breeding Laboratories. The animals were adrenalectomized 3-5 days before use and were maintained on standard Purina rat chow, 0.9% NaCl (Saline) ad libitum. [1,2,6,7 - ^{3}H [N] Dehydroepiandrosterone (97.3 Ci/mmole) was obtained from New England Nuclear. Its purity was checked by thin-layer chromatography on precoated silica sheets (Eastman, Kodak) using a benzene/ ethyl acetate (60:40 V/V) solvent system. Bovine pancrease deoxyribonuclease type I and bovine pancreas ribonuclease type A were obtained from Sigma. Trypsin, chymotrypsin, papain and wheat germ lipase were from Worthington Co. Inc. Unlabelled steroid were obtained from Sigma. All other reagents used were of analytical grade.

PREPARATION OF CYTOSOL: - Rats were killed by cervical dislocation and livers were perfused with buffer A (10mM Tris, 0.25M sucrose, pH 7.6 at 4^{o}C). The livers were removed, cleansed of adhering tissue and homogenized (1:3 W/V) in buffer A with a Teflon homogenizer. The cytosol fraction was prepared by centrifuging the homogenate at 105,000 x g for 60 min at 0^{o}C. The cytosol was carefully removed, adjusted to approximately 5mg protein/ml cytosol and a final pH of 7.6 at 4^{o}C and used. Protein was determined by the method of Lowry et al using bovine serum albumin as the standard (10). The cytosolic samples were incubated with 1-50nM of [^{3}H] DHEA in the presence or absence of 500 fold excess of unlabeled steroid. After incubation for 4h at 4^{o}C, the specific macromolecular binding was determined using the charcoal-dextran technique (11). The data were evaluated using method of Scatchard (12).

Hepatic cytosol was incubated for 4h at 4^{o}C with 10nM [^{3}H] DHEA with or without 10mM sodium molybdate or 5mM dithiothreitol or 0.3M KCl before

denaturation studies were carried out at 35°C for various times. The samples were then treated with charcoal-dextran and radioactivity was determined using Beckman Ready Solve-EP.

In vitro activation studies were carried out by preincubating cytosol with [^{3}H] DHEA for 4h at 4°C in the presence or absence of 10mM sodium molybdate. Portions of samples were then further incubated for 45min at 25°C. 200μl cytosol samples were mixed with 100μl DNA-cellulose (1.4mg native calf thymus DNA/ml cellulose) and incubated for 60min at 4°C with occasional stirring. The incubation samples were washed thrice with 1ml of buffer A. The pellets were suspended overnight in the Beckman Ready Solve - EP solvent and counted as described before (11), DNA was measured using the diphenylamine assay of Burton (13).

Linear 5-20% sucrose gradients were prepared in 10mM Tris buffer, PH 7.6 containing 30mM KCl (low salt) or 300mM KCl (high salt). Bovine Serum albumin (4.6 S) and human gamma globulin (7.1 S) were used as references to estimate sedimentation coefficients (14). This work was aided by funds from Dr. Susan Mellette and Barbara J. Johnson.

RESULTS: Scatchard plot analysis of data presented in Fig. 1 show that [^{3}H] DHEA was bound to a rat hepatic cytosolic macromolecule having a single class of binding site. With apparent dissociation constant (Kd) of about 2.3nM. Interestingly, addition of either 0.1M NaSCN or 0.3M KCl increased the concentration of the binding sites without effecting the binding affinity (Kd 2.8nM).

Steroid specificity of binding presented in Fig 2 show that [^{3}H] DHEA was displaced effectively (70-80%) with unlabelled DHEA, β etiocholanolone and androsterone. 17β estradiol, progesterone, and testosterone also considerably displaced the binding of [^{3}H] DHEA (40-50%). While dexamethasone and aldosterone were less effective.

Table I represents the effect of various enzyme treatments on the binding of [^{3}H] DHEA to the rat hepatic cytosol. The binding was markedly inhibited by trypsin and chymotrypsin. Deoxyribonuclease, ribonuclease and lipase were found to be without any effect.

The [^{3}H] DHEA - macromolecular complex was found to be stable up to 2 hrs at 35°C and the addition of 5mM dithiothreitol, 10mM molybdate or 0.3M KCl further stabilized the complex at 35°C. (Fig 3).

Data presented in Fig 4 show that pretreatment of hepatic cytosol with sulfhydryl blocking reagents such as N-ethylmalemide or iodoacetamide reduced the [^{3}H] DHEA binding up to 60-70%. Interestingly, p-

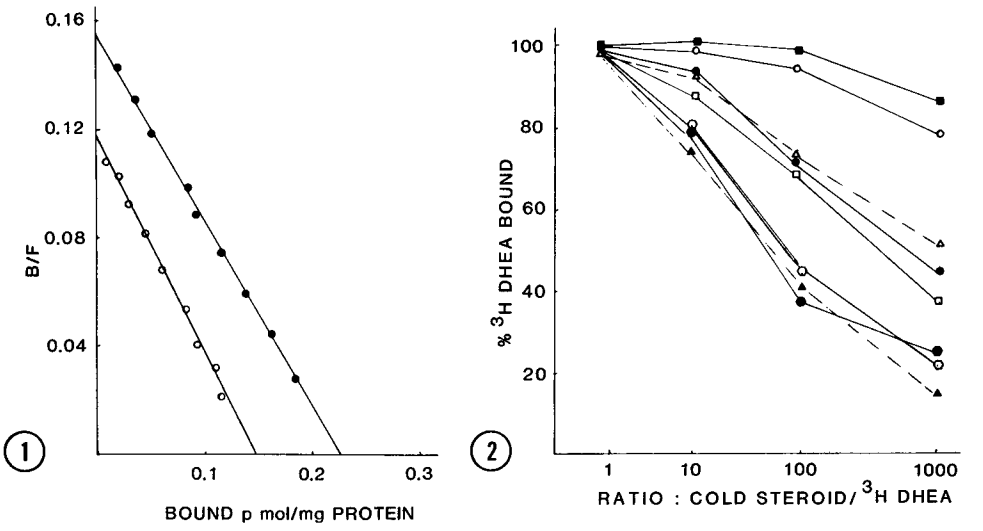


Fig 1. Scatchard analysis of specific [^{3}H] DHEA binding in rat hepatic cytosol. Binding were carreid out as described in Methods. Control (0-0) and 0.1M NaSCN (●-●).

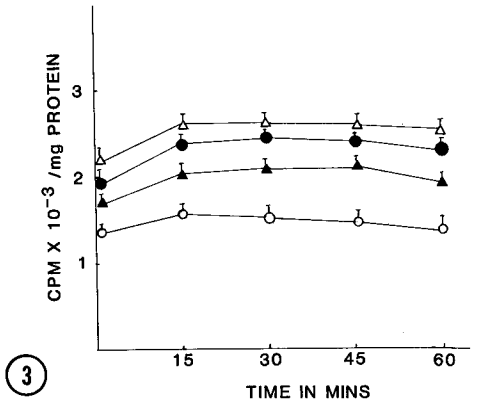
Fig 2. Steroid specificity of [^{3}H] DHEA binding. Samples in triplicate were incubated with 10nM [^{3}H] DHEA alone or in the presence of various concentrations of unlabeled steroids and specific bound radioactivity determined as described in Methods. Each point is the mean value of two experiments. Aldosterone (■-■), Dexamethasone (0-0) Progesterone (Δ-Δ), Testosterone (●-●), 17β estradiol (□), DHEA (○), Androsterone (⬣), β etiocholanolone (▲).

TABLE I

Effects of various hydrolytic enzymes on rat hepatic cytosolic [^{3}H] DHEA binding

Enzyme	[^{3}H] DHEA bound	%
Untreated control	3,680	100
Trypsin	256	7
Chymotrypsin	126	4
Lipase	3,460	96
Deoxyribonuclease	3,698	100
Ribonuclease	3,574	97

Samples were incubated for 60 min at 4°C with 0.1ml (1mg per ml) of the appropriate enzymes or with homogenization buffer. The specific binding was determined as described in Methods.



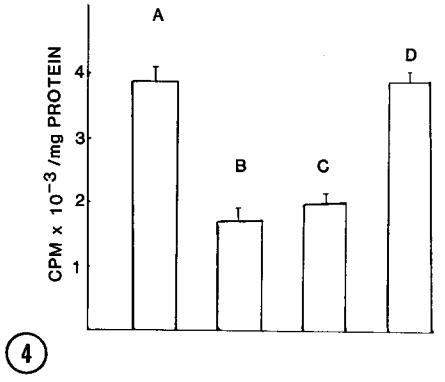


Fig 3. Stability of preformed [^{3}H] DHEA-macromolecular complex. Hepatic cytosol samples were incubated with 10nM [^{3}H] DHEA alone (0-0) or in the presence of 10mM sodium molybdate (Δ-Δ), or 5mM dithiothreitol (▲-▲) or 0.3M KCl (●-●) for 4h at 4°C. At different time intervals, triplicate aliquots were withdrawn and bound radioactivity was determined as described in Methods. Results are mean ± S.E. of four determinations.

Fig 4. Effect of - SH blocking reagents on rat liver [^{3}H] DHEA binding. Control samples (A) or samples treated with 5mM concentration of iodoacetamide (B), 5mM N-ethylmalemimide (c) or, 5mM p-chloromero-benzoate (D) for 30 min at 4°C with 10nM [^{3}H] DHEA. Iodoacetamide, N-ethylamlemide and p-chloromercuribenzoate were prepared in concentrated stock solution (100mM, pH 7.6) before use. The specific binding was determined as described in Methods. Results are mean ± S.E. of three determinations done in triplicate.

chloromercurobenzoate at 5mM concentration was unable to inhibit the [^{3}H] DHEA binding.

[^{3}H] DHEA binding was highest in the liver, followed by kidney and testis, plasma had no detectable amount of [^{3}H] DHEA binding (Table II).

The [^{3}H] DHEA binder was sedimented at 7-8 S macromolecular complex in low ionic strength sucrose density gradients and 3-4 S in high ionic strength sucrose density gradients (Fig 5).

The hepatic cytosolic [^{3}H] DHEA complex when incubated in vitro for 45 min at 25°C bound poorly to the DNA-cellulose. Only 30-40% binding over control untreated samples was noted on thermal activation (Fig 6). The addition of 10mM sodium molybdate completely inhibited DNA-cellulose binding of [^{3}H] DHEA -bound complex.

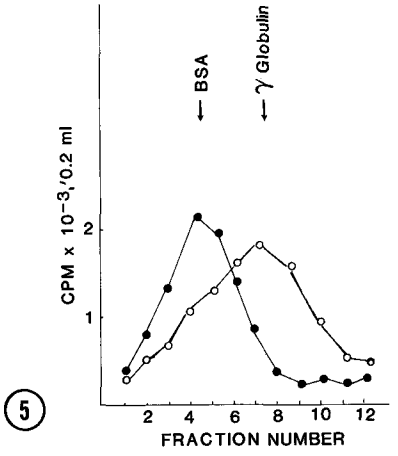
DISCUSSION: The result described here suggest the presence of high affinity, steroid specific binding for DHEA in the rat hepatic cytosol. In this

TABLE II

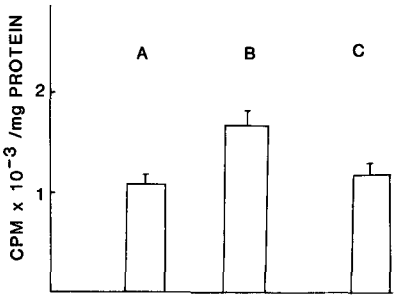
Specific [^{3}H] DHEA binding in cytosol of various tissues of rat

Tissue	Specific [^{3}H] DHEA binding cpm/mg protein
Liver	3200 ± 240
Kidney	1300 ± 148
Testis	1108 ± 96
Heart	946 ± 46
Plasma	280 ± 38

Cytosol prepared by homogenizing different tissues (1:3 W/V) in buffer A. The specific binding was determined as described in Methods. The results are mean ± S.E. of two experiments with triplicate determinations.



(5)



(6)

Fig 5. Sucrose density gradient analysis of rat liver [^{3}H] DHEA -macro - molecular complex. Cytosol saturated with 10nM [^{3}H] DHEA at 4°C for 4h and then treated with charcoal dextran. 400 µl of supernatant sample was applied to low salt (30mM KCl) (0-0) or high salt (300 mM KCl) gradients (●-●). Samples were centrifuged at 4°C for 18h in a Spinco SW rotor at 35,000 rpm. Gilford Densiflow apparatus was used to collect 8 drop samples, starting from the top of the gradients. Bovine serum albumin (BSA). The details are described in Methods.

Fig 6. DNA-cellulose binding of [^{3}H] DHEA-macromolecular complex. Hepatic cytosol samples were preincubated for 4h at 4°C in the presence of 10nM [^{3}H] DHEA (A). Samples were further incubated for 45 min at 25°C in the absence (B) or presence of (c) 10mM sodium molybdate. DNA-cellulose bound radioactivity was determined as described under Methods. Results are mean ± S.E. of three experiments done in triplicate.

regard, we found some interesting properties of the [^{3}H] DHEA binding protein: (a) [^{3}H] DHEA-macromolecular complex is very stable at 35°C. (b) Increasing the salt concentration (by addition of either 0.1M NaSCN or 0.3M KCl) of the hepatic cytosol resulted in enhanced binding of [^{3}H] DHEA to its cytosolic protein at 4°C without altering the affinity. Thus, it seems that high salt keeps DHEA binding sites in vitro in a configuration which not only enhanced its binding to DHEA, but also imparts stability to the [^{3}H] DHEA protein bound complex. Finally, (c) the [^{3}H] DHEA-macromolecular complex responds poorly to thermal activation in vitro as judged from its DNA-cellulose binding.

In order to assign any physiological revelance to the specific hepatic DHEA binding seen, we have deliberately studied the possible presence of a specific receptor protein utilizing physiologic concentrations of DHEA. In constrast, 0.4% DHEA has been commonly used in rodent feeding experiments (6,7) while from 20-80mg/kg/day have been used in clinical studies (7). In our in vitro studies μM concentration of DHEA have been used in the cell culture experiments (unpublished data), and there is a great dissociation between the in vitro affinity of DHEA reported here compared to reports of DHEA's clinical dosage or its in vivo biological activity or response. (7)

One probable explanation for in vitro and in vivo differences may be attributed to the lower in vitro thermal activation of the [^{3}H] DHEA complex. However, in addition to our observed slower activation of [^{3}H] DHEA - cytosolic complex, it is probable that absorption, distribution and more likely the in vivo metabolism of DHEA may play a crucial role in in vivo biologic responses. In that regard, it is possible that DHEA is probably converted in vivo to an active metabolite. Various researcher have attributed DHEA or DHEA-sulfate (DHEA-S) functions in vivo to its conversion into either androgenic or estrogenic compounds (15,16). As such, DHEA is described in the literature as a weak androgenic 17β ketosteroid and recent observations have shown it to have clinical androgenic effects (7). It is, therefore, important that further systematic work should be carried out using both in vivo and in vitro studies to determine whether the observed in

vitro specific [^{3}H] DHEA hepatic binding reported here is represented by interaction with distinct receptor or that DHEA and its metabolites exert their effects by binding to well characterized estrogen, androgen or progesterone receptors in order to influence various cellular function in normal and transformed cells. This is particularly important to the renewed interest in DHEA and related structures for their potential antidiabetic, antiviral, antiobesity and anticarcinogenic actions. (2,4,6,7,17).

REFERENCES

1. C. Migeon, B. Keller, B. Lawrence, et al., J. Clin. Endocrinol. Metab. 17 051-1062 (1957).
2. A. Schwartz, Cancer Res. 39 1129-1132 (1979).
3. M. Cleary, R. Seidensatt, R. Pearson, et al., Lipids 12 409-413 (1977).
4. D. Coleman, E. Leiter and R. Schwizer, Diabetes 31, 830-835 (1982).
5. C. Corpechot, P. Robel, M. Axelson et al. Brain Res. 270 119-125 (1983).
6. A. Schwartz in Molecular Biology of Aging (A. Woodhead, Ed.) Plenum Press New York, p. 181-191, 1985.
7. W. Regelson, R. Loria and M. Kalimi in Neuroimmunomodulation: Interventions in Aging and Cancer (Eds. W. Pierpaoli and N. Spector) Annals of the New York Academy of Sciences Vol. 521, The New York Academy of Sciences, New York, p. 260-273, 1988.
8. J. Thijssen, J. Poortman and J. Schwarz, J. Steroid Biochem. 6 729-734, (1975).
9. J. Poortman, J. Vroegindewey, J. Thijssen and F. Schwarz, Mol. Cell Endocrinology 8 27-34 (1977).
10. O. Lowry, N. Rosebrough, A. Farr and R. Randall, J. Biol. Chem. 193 265-275 (1951).
11. M. Kalimi, P. Colman and P. Feigelson, J. Biol. Chem. 250 1080-1086 (1975).
12. G. Scatchard, Ann. N.Y. Acad. Sci. 51 660-664 (1949).
13. D. Burton, Biochem. J. 62 315-322, (1956).
14. R. Martin and B. Ames, J. Biol. Chem. 236 1372-1379 (1961).
15. K. Li, D. Chandra, T. Food et al. Steroids 28 561-574 (1976).
16. W. Miller and A. Forrest. Lancet 28 66-868 (1974).
17. R. Loria, T. Inge, S. Cock, A. Szakal and W. Regelson, J. Medical Virology, In Press (1988).