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SPECIFIC ESTRADIOL-17β BINDING COMPONENT IN ADULT RAT KIDNEY

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SUMMARY

An estradiol-17β (E_2) binding component has been identified in adult rat kidney which exhibits characteristics of a specific receptor. Scatchard analysis of kidney cytosol revealed a single class of binding sites for E_2, having high affinity ($\sim 0.6 \times 10^{10}$ M) and limited binding capacity (12–20 fmol/mg protein). The cytosol macromolecule has a sedimentation coefficient of approximately 4S in either low or high salt (0.4 M KCl) sucrose density gradients. Competition studies using a 100-fold excess of various unlabeled steroids demonstrated greatest inhibition by various estrogens, with E_2 and DES as the most effective competitors; whereas several androgens, aldosterone and progesterone inhibited binding very little. Incubation of kidney slices with [^{3}H]-E_2 resulted in localization of the steroid in purified nuclei. Addition of a 100-fold excess of unlabeled E_2 or DES inhibited translocation of [^{3}H]-E_2 by 94 and 79% respectively, whereas testosterone, dihydrotestosterone and aldosterone exhibited only minimal inhibition on nuclear translocation of [^{3}H]-E_2 receptor. These data offer strong evidence for the existence of an E_2 receptor in rat kidney. It remains to be determined which specific cellular events are regulated by E_2 in the rat kidney.

INTRODUCTION

A number of studies have demonstrated that estrogens can influence the kidney. Thus, estrogens have been shown to increase sodium retention in several species including the human [1], dog [2] and rat [3] and the activity of several enzymes in rat kidney including 16α-hydroxysteroid dehydrogenase [4], ornithine aminotransferase [5] and alkaline phosphatase [6]. In addition, chronic administration of E_2 induces renal tumors in hamsters [7]. If the various effects of estrogens are the result of their direct action on the kidney, it is essential to identify E_2 receptors in that organ and, indeed, several papers have demonstrated E_2 receptors in the kidney of the rat [3], mouse [8], guinea-pig [9], hamster [10, 11] and human [12].

The mammalian kidney is capable also of directly metabolizing, conjugating and excreting various estrogens; the extent of this capacity varies with the particular estrogen incubated or introduced, and with the species of animals being examined [13–19].

It has not been determined what (if any) relationship(s) exists between the presence of a specific receptor for E_2 (or a specific receptor for any other steroid) and the capacity of that kidney to metabolize, conjugate and/or excrete this steroid. In an attempt to determine whether an interrelationship exists, the adult rat kidney was used as a model. The first step in the endeavor was the further characterization of the E_2 receptor in male rat kidney. The results of this study are reported in the present paper.

MATERIALS AND METHODS

Animals used. Adult Wistar male rats were obtained from Woodlyn Laboratories, Ontario, Canada. They weighed between 350–550 g and were castrated 3–7 days (unless specified otherwise) prior to use. Animals were given tap water and Purina rat chow *ad libitum*.

Chemicals used. [2, 4, 6, 7-^{3}H]-estradiol-17β (98–115 μCi/nmol) was purchased from New England Nuclear Corp. The purity of the radioactive steroid was checked by instant thin layer chromatography (Gelman, ITLC-SA) and by Sephadex LH-20 chromatography (Pharmacia). Non-radioactive steroids, *i.e.* estrone, E_2, estriol, diethylstilbestrol (DES), testosterone, dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3α-diol), aldosterone and progesterone were purchased from Steraloids, Inc. Triton X-100 and bovine serum albumin were from Sigma. Medium-199 was purchased from Grand Island Biological Corp. The following buffers (all pH 7.5 at room temp.) were used. TE buffer (0.02 M Tris, 1.5 mM EDTA); STKM buffer I (0.32 M sucrose, 0.02 M Tris, 0.025 M KCl, 2 mM $MgCl_2$); STKM buffer II (2.1 M sucrose, 0.02 M Tris, 0.025 M KCl, 2 mM $MgCl_2$).

Preparation of kidney cytosol. Animals were anesthetized with ether, kidneys quickly removed and immersed in ice-cold physiological saline. The kidneys were then cut into several small pieces, and after washing three times with 25 ml physiological saline, homogenized in 3 vol. TE buffer using a Brinkman Polytron. Homogenates were centrifuged at 105,000 ***g***

for 1 h using a refrigerated Beckman ultracentrifuge. The upper lipid layer was discarded and the clear cytosol which contained 16–20 mg protein/ml was used for binding studies.

Measurement of [^{3}H]-estradiol-17β binding in cytosol. Scatchard analysis was performed on 0.5 ml cytosol with varying concentrations of labeled steroid (0.2–4.0 nM). Parallel incubations containing a 100-fold excess DES at each concentration of label were run to measure specific binding. Incubations were carried out for 3 h at 0–4°C with periodic gentle agitation. Free (unbound) steroid was separated from bound by passing cytosols through Sephadex G-25 columns. Collected fractions were counted directly in 10 ml of ACS scintillation fluid (Amersham). In studies examining the effect of time of incubation and castration on specific binding, 0.5 ml cytosols were incubated with 2 nM [^{3}H]-E_2 only and with same concentration of label plus 100-fold excess DES. For competition studies, 0.5 ml cytosol was incubated with 2 nM [^{3}H]-E_2 only and with the same concentration of label plus 100-fold excess of the various unlabeled steroids specified for 3 h at 0–4°C.

Sucrose density gradients of kidney cytosol. To measure sedimentation coefficients of [^{3}H]-E_2-cytosol macromolecule complex, 0.5 ml cytosol was incubated with 2 nM [^{3}H]-E_2 only and with 100-fold excess unlabeled DES. The total incubation volume was layered over a 13 ml 5–20% linear sucrose density gradient in TE buffer (low salt) and the tubes centrifuged in an SW-41 rotor at 205,000 *g* for 20 h using a refrigerated Beckman ultracentrifuge. Fractions of 10 drops were collected and counted directly in 10 ml ACS scintillation fluid. In duplicate incubations, free steroid was separated from bound by passing incubated cytosols through Sephadex G-25 column; fractions representing bound steroid were pooled, and a 0.5 ml aliquot layered over a 5–20% linear sucrose gradient. In another series of incubations cytosols were made 0.4 M with KCl and then incubated with 2 nM [^{3}H]-E_2 only and with 100-fold excess unlabeled DES. Incubated cytosols were then directly layered over 0.4 M KCl sucrose gradients (high salt) or after free steroid was separated from bound as described for low salt sucrose density gradients. Bovine serum albumin was used as a marker to determine sedimentation coefficients, following the method of Martin and Ames[20].

Localization of [^{3}H]-estradiol-17β in isolated kidney nuclei. Decapsulated rat kidneys from 3 rats were sliced to uniform thickness of approximately 2 mm using a Thomas tissue slicer. At least a single slice from each kidney was placed in each 50 ml beaker to be incubated; each beaker contained 5–6 slices of tissue, approximately 0.8–1.0 g wet weight. Kidney slices were washed three times with 25 ml ice-cold physiological saline, then preincubated for 5 min in 5 ml of medium-199 at 37°C in a rotary shaker bath. The incubation medium was removed and 10 ml fresh medium 199 was added. Tissue slices were preincubated for an additional 5 min at which time either 2 nM of [^{3}H]-E_2 only or the same concentration of labeled E_2 plus 100-fold excess of unlabeled DES, E_2, testosterone, DHT or aldosterone were added. Samples were incubated for 1 h at 37°C with constant gentle shaking. Reaction was stopped by placing beakers in an ice bath and the tissue slices washed three times with 25 ml ice-cold physiological saline and once with 10 ml STKM buffer I. Tissue slices were homogenized in 10 ml of STKM buffer I using a 30 ml glass-Teflon Potter–Elvehjem homogenizer. Homogenates were filtered through cheese cloth and then centrifuged at 800 *g* for 10 min in a refrigerated Sorvall centrifuge. The crude nuclear pellet was washed twice with 10 ml STKM buffer I. The washed crude nuclear pellet was resuspended in 1 ml STKM buffer I and this suspension was layered over an 11 ml cushion of STKM buffer II. The tubes were centrifuged at 105,000 *g* for 1 h in a refrigerated Beckman ultracentrifuge using an SW-41 rotor to isolate nuclei. The nuclear pellet was resuspended in 10 ml STKM buffer I containing 0.1% Triton X-100 and allowed to sit in cold for 10 min. Tubes were centrifuged at 1000 *g* for 15 min., and the nuclear pellet washed twice with 10 ml STKM buffer I only. The washed nuclei were resuspended in 2 ml cold STKM buffer I and extracted four times with 2 ml methylene chloride. The pooled methylene chloride extracts were counted in 10 ml ACS scintillation fluid.

Cytosol and nuclear proteins were determined by the method of Lowry *et al.*[21] using bovine serum albumin as standard. DNA was determined by the method of Burton[22] using calf thymus DNA as standard. The protein/DNA ratio for purified kidney nuclei ranged between 1.4–1.6.

RESULTS

Scatchard analysis of [^{3}H]-estradiol-17β binding to kidney cytosol component

Figure 1 represents binding studies where a constant volume (0.5 ml) of kidney cytosol was incubated with increasing concentrations (0.2–4.0 nM) of [^{3}H]-E_2; at each steroid concentration parallel incubations contained 100-fold excess DES to measure specific binding. Scatchard analysis of the data yielded a straight line indicating a single class of binding sites. From the slope of this line the apparent association constant (K_a) was $\sim 0.6 \times 10^{10}$ M. The number of binding sites was approximately 13 fmol/mg protein and ranged from 12–20 fmol/mg protein in three experiments. The inset shows the same data, but with total binding, specific binding and nonspecific binding plotted as a function of the free steroid concentration. Under the incubation conditions used, the specific binding sites reach saturation at approximately 1 nM of [^{3}H]-E_2.

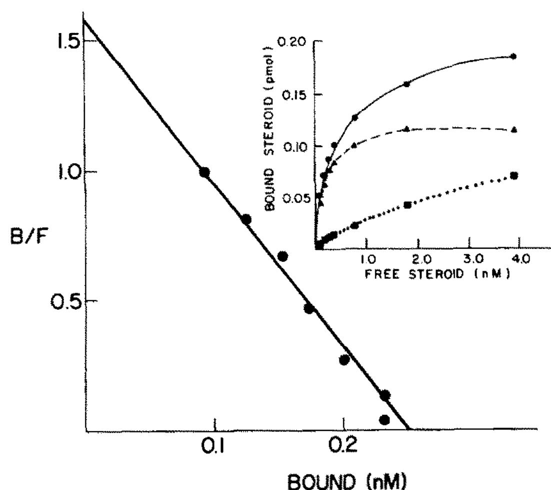

Fig. 1. Scatchard analysis of specific binding of [^{3}H]-E_2 to kidney cytosol component. Steroid concentrations ranged from 0.2–4.0 nM. At each steroid concentration tubes containing 100-fold excess unlabeled DES were included also to measure specific binding. Volume of cytosol used was 0.5 ml. Incubation was carried out at 0–4°C for 3 h. Free steroid was separated from bound by passing cytosols through Sephadex G-25 columns. Inset represents the same data but compares total steroid bound (●——●), specific binding (▲——▲) and nonspecific binding (■·····■) vs. free steroid concentrations.

Effect of incubation time on [^{3}H]-E_2 binding to kidney cytosol components

To examine the effect of incubation time on [^{3}H]-E_2 binding to kidney cytosol macromolecules, 0.5 ml of cytosol was incubated with 2 nM labeled steroid only and with 100-fold excess of unlabeled DES (Fig. 2). Samples were incubated for designated time periods at 0–4°C. Total specific binding increases sharply between 0–2 h incubation, approaches equilibrium at approximately 3 h and remained constant for 22 h, but dropped slightly by 30 h of incubation.

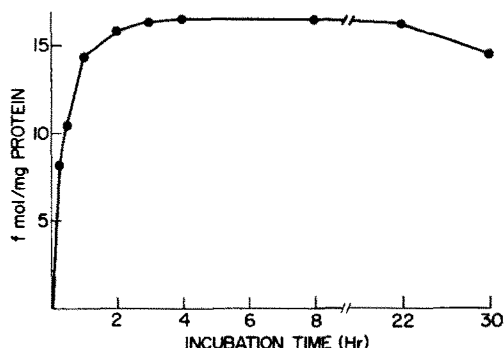

Fig. 2. Effect of incubation time on specific binding of [^{3}H]-E_2 to kidney cytosol components. 0.5 ml kidney cytosol was incubated with 2 nM [^{3}H]-E_2 at 0–4°C for varying periods of time. For each time period tubes containing 100-fold excess unlabeled DES was also incubated to determine specific binding.

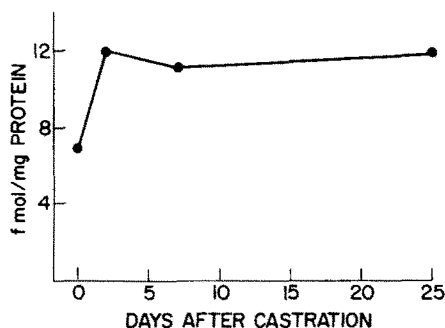

Fig. 3. Effect of castration on specific binding of [^{3}H]-E_2 to kidney cytosol component. Measurement of specific binding was performed at the same time on kidney cytosol obtained at various times after castration. Cytosol volume was 0.5 ml and concentration of label 2 nM. Incubation was performed at 0–4°C for 3 h. Specific binding was measured by also incubating tubes containing a 100-fold excess of DES.

Effect of castration on [^{3}H]-E_2 binding to kidney cytosol components

The effect of castration on specific [^{3}H]-E_2 binding to rat kidney cytosol components is presented in Fig. 3. Specific binding was lowest in kidneys obtained from intact rats, indicating some degree of occupation of binding sites by endogenous estrogens. Specific binding increased by 3 days following castration and did not change at days 7 and 25 after castration.

Sucrose density gradients of rat kidney cytosol incubated with [^{3}H]-E_2

Sedimentation patterns of [^{3}H]-E_2 binding to kidney cytosol components in 5–20% linear sucrose density gradients are given in Fig. 4. In panel A, 0.5 ml cytosol was incubated with 2 nM [^{3}H]-E_2 only (——) and with 100-fold excess DES (-----) to measure specific binding. After separation of free steroid from bound, 0.5 ml aliquots of the pooled bound fractions were layered over 5–20% linear sucrose density gradients prepared in TE buffer only (low salt). In panel B, incubation conditions were similar except cytosols were made 0.4 M with KCl, and after free steroid was separated from bound an 0.5 ml aliquot was layered over a 0.4 M KCl sucrose gradient (high salt). The arrow marks the bovine serum albumin peak. Thus, in either low or high salt sucrose gradients [^{3}H]-E_2-cytosol binding component complex sediments in the 4S region. When cytosols were applied directly to sucrose gradients (without separation of free from bound steroid) similar sedimentation patterns were observed, except a large free steroid peak often masked much of the 4S peak.

The binding capacity of the 4S [^{3}H]-E_2-binding component in low salt sucrose gradient was 14.8 ± 1.8 fmol/mg protein (ave. of 3 expts.). This compares favorably with the binding capacity of the E_2 binder measured by Scatchard analysis. Although we did not

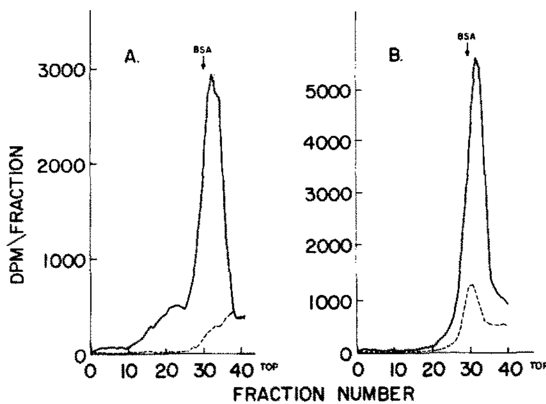

Fig. 4. Sucrose density gradients of rat kidney cytosols incubated with [^{3}H]-E_2. Panel A. Cytosol (0.5 ml) was incubated with 2 nM [^{3}H]-E_2 (——) and 2 nM [^{3}H]-E_2 plus 100-fold excess DES (-----) for 3 h at 0–4°C. Free steroid was separated from bound by Sephadex G-25 column chromatography and 0.5 ml aliquot of pooled bound counts layered over 13 ml of 5–20% linear sucrose density gradient. Panel B. Cytosol was made 0.4 M with KCl and 0.5 ml was incubated with 2 nM [^{3}H]-E_2 (——) and with 2 nM [^{3}H]-E_2 plus 100-fold excess of DES (-----). Free steroid was separated from bound by Sephadex G-25 equilibrated with 0.4 M KCl, and 0.5 ml aliquots of pooled bound counts were layered over 13 ml of 5–20% linear sucrose gradients prepared in 0.4 M KCl. Arrow marks bovine serum albumin marker.

observe an 8S receptor under low salt conditions as was reported in mouse kidney [8], our results of a 4S binder only are similar to those reported in non-malignant hamster kidney [11]. The significance of these differences in receptor size is not clear.

Steroid specificity of [^{3}H]-E_2 binding to kidney cytosol components

The effectiveness of various unlabeled steroids to compete with [^{3}H]-E_2 for specific binding sites in kidney cytosol is presented in Table 1. As a group the estrogens inhibited binding of label to a highest degree, among which E_2 and DES exhibited greatest competition. Each of the androgens, aldosterone and progesterone exhibited minimum inhibition of binding.

Table 1. Specificity of [^{3}H]-estradiol-17β binding to rat kidney cytosol

Competing steroid	fmol/mg protein	% of Control
None (control)	14.2	100
Estrone	6.6	47
Estradiol-17β	4.6	32
DES	5.8	40
Estriol	7.8	55
Testosterone	11.5	81
DHT	11.9	84
5α-androstane 3α, 17β-diol	11.4	81
Aldosterone	12.8	90
Progesterone	12.4	88

0.5 ml cytosol was incubated with 2 nM [^{3}H]-E_2 only and with 100-fold excess of various unlabeled steroids. Incubation was carried out at 0–4°C for 3 h, and free steroid was removed from bound by Sephadex G-25 chromatography.

Table 2. Specificity of [^{3}H]-estradiol-17β localization in isolated rat kidney nuclei

Competing steroid	fmol/mg DNA	% of Control
None (control)	85.9	100
DES	18.5	21
Estradiol-17β	4.7	6
Testosterone	59.9	70
DHT	63.6	74
Aldosterone	63.4	74

Rat kidney slices were incubated in 10 ml medium-199 with 2 nM [^{3}H]-E_2 only and with 100-fold excess of various unlabeled steroids. Nuclei were isolated (see Materials and Methods for details) and extracted with methylene chloride.

Localization of [^{3}H]-estradiol-17β in isolated rat kidney nuclei

The results demonstrating the specificity of [^{3}H]-E_2 localization in isolated nuclei obtained from incubated rat kidney slices are presented in Table 2. The highest concentration of label was observed when kidney slices were incubated with 2 nM [^{3}H]-E_2 only. A 100-fold excess of both unlabeled E_2 and DES dramatically reduced the amount of [^{3}H]-E_2 localized in nuclei. The fact that testosterone, DHT and aldosterone inhibited the localization of [^{3}H]-E_2 in nuclei much less than did E_2 and DES points to the specificity of [^{3}H]-E_2 translocation in rat kidney nuclei.

DISCUSSION

Evidence has been presented for the existence of a cytosol binding component in adult male rat kidney that specifically binds E_2. This cytosol estrogen binder exhibits both high affinity ($K_a \sim 0.6 \times 10^{10}$ M) and limited binding capacity (12–20 fmol/mg protein) for this steroid. It sediments in the 4S region of either low salt or high salt sucrose gradients. Estrogens, especially E_2 and DES, compete effectively for the cytosol binder, but various androgens, aldosterone and progesterone compete only slightly. There is evidence also for specific translocation of the [^{3}H]-E_2-cytosol complex into the nucleus. This cytosol binding component thus exhibits characteristics of a specific receptor for E_2.

These results confirm and extend the earlier report by DeVries *et al.* (1972) on the identification of an E_2 receptor in adult male rat kidney and support similar results in the kidney of humans [12] and hamsters [10, 11]. However, it remains to be determined whether E_2, after binding to its receptor, directly alters nuclear transcription and thereby influences the

excretion of sodium by kidney [1–3] or directly stimulates synthesis of specific enzymes [4–6].

Even though the essential role of specific receptors for steroids in eliciting a specific effect in "target organs" is acknowledged, a heretofore unexplored question is the relationship between the existence of specific receptors exhibiting high affinity and limited capacity for a steroid and the extent to which this steroid is metabolized or conjugated in the organ. Using the rat kidney as a model and having established the presence of a specific E_2 receptor in it, we are currently examining the *in vitro* and *in vivo* capacity of rat kidney to metabolize and/or conjugate estrogens. Ultimately, we wish to identify the specific anatomic areas in the kidney where the receptors and metabolizing and conjugating enzymes are localized.

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