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16α,18-DIHYDROXYDEOXYCORTICOSTERONE AND THE BINDING OF ALDOSTERONE TO MINERALOCORTICOID RECEPTORS IN KIDNEY OF ADRENALECTOMIZED RATS

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SUMMARY

It has been recently suggested that 16α,18-dihydroxydeoxycorticosterone (16α,18-diOHDOC) may act as a "positive allosteric effector" of the binding of aldosterone to mineralocorticoid receptors. To test this hypothesis, a series of *in vitro* and *in vivo* studies examining the effect of 16α,18-diOHDOC on tritiated aldosterone (^{3}HA) binding to mineralocorticoid receptors was performed. Using kidney slices from adrenalectomized rats, *in vitro* incubations were made for 20′ at 37C, over a range of concentrations of ^{3}HA plus tenfold dexamethasone to confine tracer binding to mineralocorticoid receptors. At no concentration of ^{3}HA did 16α,18-diOHDOC enhance binding; at all tracer concentrations a slight competing effect was observed. When ^{3}HA was injected into rats *in vivo* with and without 16α,18-diOHDOC, a similar insignificant displacement of ^{3}HA binding was seen in renal cytoplasmic fractions from adrenalectomized test rats.

Additional *in vitro* studies were performed in an attempt to elucidate the mechanism of postulated action of 16α,18-diOHDOC. Neither renal cytoplasmic binding of oestradiol, postulated as a secondary pathway for steroid influenced Na^+ retention, nor the binding of dexamethasone to renal glucocorticoid receptors, was altered by 16α,18-diOHDOC. Binding of tritiated 16α,18-diOHDOC in renal cytoplasmic fractions was shown to be non specific, in that it could not be displaced by excess unlabelled 16α,18-diOHDOC.

Finally, in a series of *in vivo* experiments using adrenalectomized rats, we could not show any effect of 16α,18-diOHDOC on urinary electrolyte excretion, either alone or in combination with low doses of aldosterone.

Accordingly, we can find no evidence for 16α-diOHDOC having a direct effect on the kidney: in particular, there would appear as yet no molecular evidence for 16α,18-diOHDOC being a positive allosteric effector of aldosterone.

INTRODUCTION

Within the diagnostic classification of essential hypertension there have been variously [1, 2] claimed to be separable a group of patients with renin levels that are subnormal and respond sluggishly, if at all, to provocative stimuli. Such patients differ from those with Conn's Syndrome in that their levels of circulating aldosterone, as routinely measured, lie within normal limits [3]. In an attempt to reconcile these findings considerable attention has been focussed on the search for other abnormalities of adrenal secretion: candidates proposed for such a role have included deoxycorticosterone (DOC), 18-hydroxydeoxycorticosterone (18-OH DOC), 16β-hydroxydehydroepiandrosterone (16βOH-DHEA) and 16α,18-dihydroxydeoxycorticosterone (16α,18-diOHDOC) [4–7].

For the first three of these steroids the proposed mechanism of action was as mineralocorticoid *per se*, with abnormally high levels (in a proportion of patients with low renin hypertension) being the cause of fluid and electrolyte retention outside normal feedback control. 16α,18-diOHDOC, on the other hand, has been shown [7] to have no inherent salt-retaining action. Its postulated role in abnormal salt retention is that of enhancing the antinatriuretic effect of aldosterone, as demonstrated in the rat bioassay for urinary electrolyte activity. Upon the basis of the *in vivo* effect on urinary electrolytes, 16α,18-diOHDOC has been proposed as a "positive allosteric effector" of aldosterone at the receptor level, increasing the affinity of the renal mineralocorticoid receptors for aldosterone. A similar hypothesis has been advanced for the potentiation of androgen effects upon the kidney observed with low doses of certain progestational steroids [8]. The series of experiments to be detailed was made to examine, at the level of the renal cytoplasmic receptor, whether or not the reported *in vivo* effects of 16α,18-diOHDOC can be explained on the basis of a positive cooperative effect.

MATERIALS AND METHODS

Tritiated aldosterone (^{3}HA, 50 Ci/mmol), tritiated oestradiol ($^3HE_2$, 50 Ci/mmol) and tritiated dexamethasone (^{3}HDM, 27 Ci/mmol) were purchased from Amersham–Searle (UK). Tritiated 16α-18-diOHDOC (^{3}H 16α,18-diOHDOC, 50 Ci/mmol), and unlabelled 16α,18-diOHDOC were kindly supplied by Dr. James Melby, Boston, Mass. Unlabelled dexamethasone was the gift of Merck, Sharp & Dohme (Aust.); other unlabelled steroids used were chromatography grade and purchased from Calbiochem, Los Angeles.

Mature Sprague–Dawley rats of either sex weighing between 120 and 180 g were maintained after bilateral adrenalectomy on standard chow and 0.9% saline until used for binding experiments 3–7 days later. Those used in the *in vivo* bioassay experiments were adrenalectomized the day before use. Immediately after adrenalectomy bioassay rats were given an intraperitoneal injection of 1 ml/100 g of a mixture of 0.9% NaCl plus 2% ethanol containing dexamethasone 20 μg/ml. Overnight no food, but free access to 0.9% NaCl, was provided. The following morning at zero time the bladder was emptied by suprapubic pressure, each animal given 3 ml/100 g of water by gavage, and the urine collected over the period 0 to 1 h. At time +1 h the test substances were injected in 4% ethanol in 0.9% NaCl, 1 ml/100 g i.p. At time + 2 h urine was expressed and discarded, and the animals given a further 3 ml/100 g of water by gavage. Urine was collected from +2 to +5 h; the Na^+/Cr and K^+/Cr ratios were determined in the 0–1 hour and 2–5 h urines. Na^+ and K^+ values were determined using an IL 243 flame photometer with an internal Li^+ standard. Creatinine values were determined with a Technicon autoanalyser.

Steroid binding studies were effected either in renal slices (^{3}HA, ^{3}HDM) or in preformed renal cytoplasmic extracts, (^{3}H-E_2, ^{3}H-16α,di-OHDOC). For the slice studies, animals were exsanguinated under pentobarbitone anaesthesia and perfused with ice-cold saline *via* the abdominal aorta: the kidneys were removed, bisected and placed in ice-cold incubating solution (Na^+ 133 K^+ 6 Cl^- 134 H_2PO4^- 6 Ca^{2+} 1 Mg^{2+} 0.5 Tris–HCl 5 Glucose 5 (all mM; pH 7.4)). Slices of 230 μm thickness were made with a Sorvall tissue chopper, the slices from 4–6 animals pooled for each experiment, and the pool divided into the appropriate number of aliquots.

Slices were incubated with tritiated steroid in the presence or absence of unlabelled 16α,18-diOHDOC: in all experiments an equivalent number of incubations were run in the presence of ⩾500 fold unlabelled tracer, to determine the non-displaceable binding. In slice experiments using ^{3}HA aldosterone as tracer 10 fold unlabelled DM was included in all flasks, to confine ^{3}HA binding to mineralocorticoid receptors as has been reported previously [9].

At the end of the period of incubation the slices were drained, homogenized in 0.25 M sucrose-3 mM Tris–HCl for three seconds with a Polytron P10 (speed 2), and the homogenate centrifuged at 30,000*g* for 30 min to yield a high speed supernatant (HSS) containing cytoplasmic steroid receptors. Aliquots of 1 ml vol. of this HSS were passed through G-50 (fine) Sephadex minicolumns of bed vol. 3.6 ml to separate protein bound from residual free steroid. This procedure has been extensively employed previously in similar studies [10] and its validation recently documented in detail [11]. Aliquots of the external volume of the Sephadex minicolumns were counted in a Packard 3375 using 1 ml of aqueous solution with 10 ml of counting solution (1 litre toluene, 500 ml Teric X 10, 0.15 g POPOP 2.75 g PPO). Protein concentrations were determined by the method of Warburg and Christian[12].

In the studies made on renal cytoplasmic extracts, kidneys were chopped fine, homogenized, and the homogenate centrifuged for 30 min at 30,000*g*. Aliquots of HSS were incubated with tritiated steroid ± unlabelled steroid for 90 min at 4 C, at the end of which period of incubation separation of bound from free steroid, and subsequent steps, were as described above.

RESULTS

Figure 1 shows the effect of 16α,18-OHDOC, at a concentration of 2×10^{-7}M, on the cytoplasmic binding of ^{3}HA. The K_d (37C) of mineralocorticoid receptors in rat kidney has been previously established to be 5×10^{-10}M [9]. A range of concentrations of ^{3}HA, spanning this half-saturating concentration, was used, plus tenfold dexamethasone to confine tracer binding to mineralocorticoid receptors. In the presence of 16α,18-diOHDOC in 200-800 fold excess, the binding of ^{3}HA is not potentiated: the steroid appears to be a weak competitor for mineralocorticoid receptors.

The lack of *in vitro* effect of 16α,18-diOHDOC does not preclude the possibility that the steroid, itself inactive, is converted to an active metabolite *in vivo*. To examine this possibility a series of *in vivo* studies examining the effect of 16α,18-diOHDOC on ^{3}HA binding was made. Renal cytoplasmic binding of ^{3}HA injected subcutaneously 20 min before sacrifice is shown in Fig. 2. The mean values of six rats injected with ^{3}HA 5×10^{-11} mol is shown as 100%. Con-

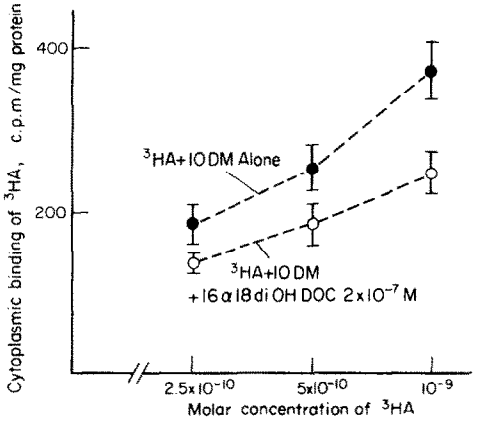

Fig. 1. Effect of 16α,18-diOHDOC 2×10^{-7}M on the binding of tritiated aldosterone (^{3}HA) to mineralocorticoid receptors. Kidney slices from adrenalectomized rats were incubated for 20 min at 37C with ^{3}HA plus tenfold unlabelled dexamethasone in the presence or absence of 16α,18-diOHDOC. An equal number of incubations were made in the presence of a ⩾500 fold excess of unlabelled DOC to determine non-displaceable binding, which was less than 10% at all concentrations of ^{3}HA used, and has been subtracted from the values shown in the figure. Values shown are the means ± SEM of at least six determinations at each concentration.

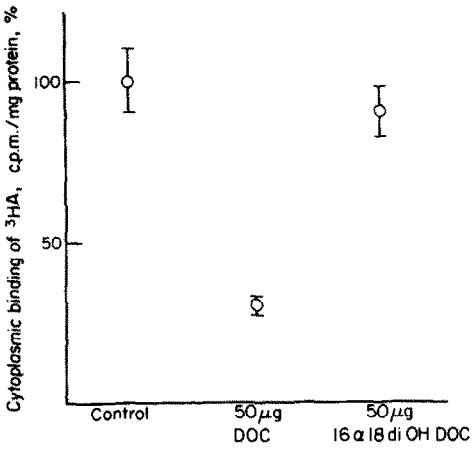

Fig. 2. Effect of 16α,18-diOHDOC on binding of tritiated aldosterone (^{3}HA) to mineralocorticoid receptors *in vivo*. Adrenalectomized rats were injected subcutaneously with 5×10^{-11} mol of ^{3}HA either alone, with 50 μg DOC, or with 50 μg 16α,18-diOHDOC. After twenty minutes animals were exsanguinated and perfused via the abdominal aorta with 20 ml of ice-cold saline. Renal high speed supernatants were prepared and the bound ^{3}HA separated from residual free by Sephadex chromatography. Values shown are the means ± SEM of six rats for each point.

comitant injection of 50 μg DOC reduces binding of ^{3}HA to 30% of control values. Concomitant injection of 50 μg 16α,18-diOHDOC, as in the *in vitro* experiments, did not enhance ^{3}HA binding.

The lack of effect of 16α,18-diOHDOC on ^{3}HDM binding in kidney slices is shown in Fig. 3. Similarly, 16α,18-diOHDOC appears (Fig. 4) without significant effect upon the renal binding of $^3HE_2$, posited as a secondary pathway for steroid modulation of Na^+ excretion [13]. To investigate the possibility that 16α-18-diOHDOC acts *via* its 'own' receptors, rather

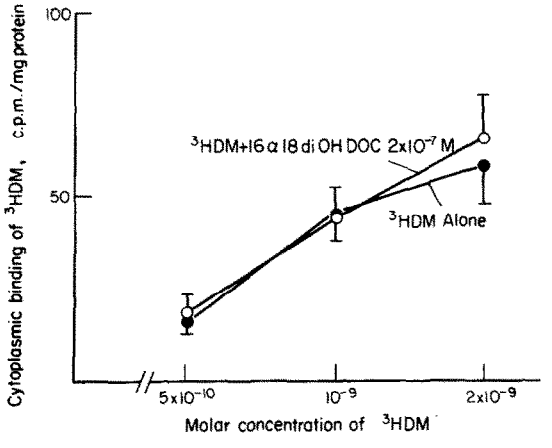

Fig. 3. Effect of 16α,18-diOHDOC 2×10^{-7}M on the binding of tritiated dexamethasone (^{3}HDM) to glucocorticoid receptors. Kidney slices from adrenalectomized rats were incubated for 20 min at 37C with ^{3}HDM in the presence or absence of 16α,18-diOHDOC. An equal number of incubations was done in the presence of ⩾ 5000 fold excess of unlabelled DM to determine non-displaceable binding, which was less than 10% at all concentrations of ^{3}HDM used, and has been subtracted from the values shown in the figure. Values shown are the means ± SEM of at least four determinations at each concentration.

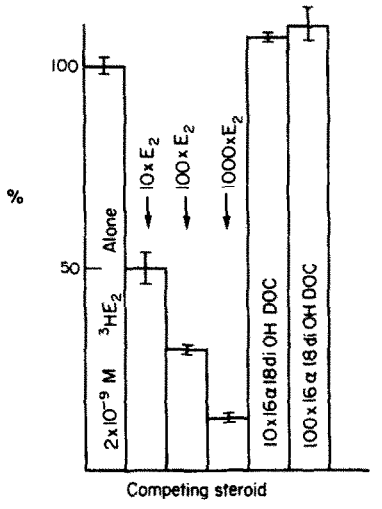

Fig. 4. Effect of 16α,18-diOHDOC on the binding of tritiated oestradiol ($^3HE_2$). Cytosol prepared from the kidneys of adrenalectomized rats was incubated for 3 h at 4C with $^3HE_2$ 2×10^{-9} M either alone, with 10, 100 or 1000 fold unlabelled E_2, or with 10 or 100 fold unlabelled 16α,18-diOHDOC. Values shown are the mean ± SEM of duplicate determinations in three experiments.

than influencing mineralocorticoid, glucocorticoid or oestrogen receptors, renal cytosol was incubated with ^{3}H-16α,18-diOHDOC 10^{-8}M either alone or in the presence of a variety of unlabelled steroids. Binding of ^{3}H-16α,18-diOHDOC was low (< 2 picomol/mg cytosol protein) and minimally displaceable either by unlabelled 16α,18-diOHDOC or any of the other steroids used (Fig. 5). Accordingly there appears to be no demonstrable high affinity, limited capacity binding of ^{3}H-16α,18-diOHDOC to sites with characteristics appropriate for physiological receptors.

In a series of *in vivo* experiments shown in Fig. 6, the effect upon urinary K^+ and Na^+ excretion of 16α,18-diOHDOC was examined. Groups of ten rats (each 7♀, 3♂) were challenged with either vehicle

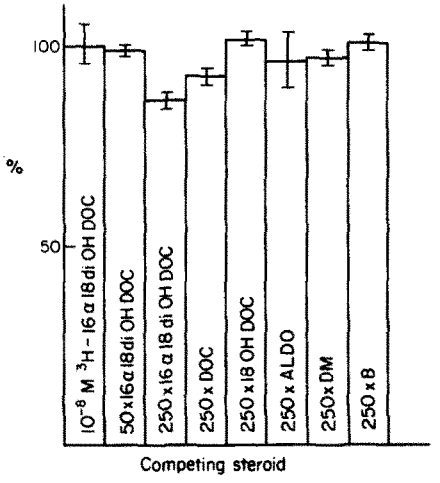

Fig. 5. Binding of ^{3}H-16α,18-diOHDOC in renal cytosol prepared from adrenalectomized rats. Renal cytosol was incubated for 3 h at 4C with 10^{-8}M ^{3}H-16α,18-diOHDOC either alone, or with concentrations as indicated of competing unlabelled steroids. Values shown are the mean ± SEM of duplicate determinations in three experiments.

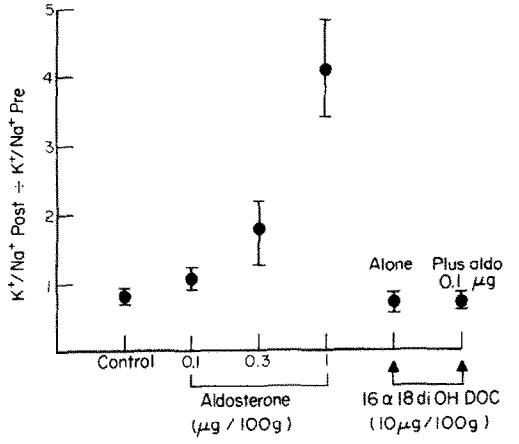

Fig. 6. *In vivo* assay for electrolyte activity of 16α-18-diOHDOC. Adrenalectomized rats were given intraperitoneal injections of either vehicle alone (4% ethanol in 0.9% NaCl), or aldosterone and or 16α,18-diOHDOC at the concentrations indicated. The results are plotted as the urinary K^+ Na^+ ratio in the period +1 to +4 h after injection divided by the urinary K^+ Na^+ ratio during the period −1 to 0 h. Values shown are the mean ± SEM of ten rats per group.

alone, aldosterone 0.1, 0.3 or 1.0 μg/100 g, and 16α,18-diOHDOC 10 μg ± aldosterone 0.1 μg/100 g. No consistent differences within the groups between sexes were noted. In this series of experiments 16α,18-diOHDOC did not potentiate the effect of 0.1 μg aldosterone. The lack of effect shown in Fig. 6 remains equally the case if the electrolyte effects are expressed in terms of sodium excretion alone, without taking potassium into account.

DISCUSSION

The results of the series of experiments detailed above can be summarized as follows: First, 16α,18-diOHDOC appears to have negligible affinity for renal mineralocorticoid, glucocorticoid or oestrogenic receptors *in vitro*. Secondly, neither *in vitro* nor *in vivo* does 16α,18-diOHDOC potentiate the binding of tritiated aldosterone to renal mineralocorticoid receptors. In addition, the steroid itself does not appear to have receptors in the kidney distinct from those for mineralocorticoids, glucocorticoids or oestrogenic steroids. Finally, in a limited series of *in vivo* bioassay experiments, 16α,18-diOHDOC did not enhance the salt retaining effect of a low dose of administered aldosterone.

In the preliminary experiment made by one of the authors (JWF) at L'Hopital Necker, Paris (quoted in [7]) 16α,18-diOHDOC appeared to potentiate the binding of tritiated aldosterone. Subsequent experiments in Paris, and those reported here done in Melbourne, showed no increased binding.

The steroid modulation of renal electrolyte handling is undoubtedly more complex than is commonly acknowledged. Examples of this complexity are the reported paradoxical natriuretic action of aldosterone in Na^+ loaded sheep [14], which action can be reversed by prolactin; the Na^+ retaining but not the kaliuretic effect of aldosterone being abolished by actinomycin D [15–16]; and the prevention of the kaliuretic effects of large doses of DOCA by the simultaneous administration of progesterone [17]. Accordingly it would be imprudent to dismiss 16α,18-diOHDOC as necessarily without a role in Na^+ homeostasis. The bioassay results reported by Melby and Dale[7] have been confirmed by a second group (Melby, personal communication). We have no ready explanation why 16α,18-diOHDOC was without effect in the methodologically different, but we believe equally valid, bioassay used in our studies.

Our conclusion would be, therefore, a tentative one: that if 16α,18-diOHDOC affects Na^+ handling, it does so only under certain conditions, which are as yet undefined; and that this effect would not appear mediated via *renal* receptors for mineralocorticoids, glucocorticoids, oestrogens or 16α,18-diOHDOC itself. In particular, we can find no evidence for a positive cooperative effect on aldosterone binding by renal mineralocorticoid receptors.

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REFERENCES

1. Kuchel O., Fishman L. M., Liddle G. W. and Michelakis A. M.: *Ant. Int. Med.* **67** (1967) 791–799.
2. Helmer O. M. and Judson W. E.: *Circulation* **38** (1968) 965–976.
3. Crane M. G.: *Ann. Int. Med.* **79** (1973) 411–424.
4. Brown J. J., Ferriss J. B., Fraser R., Lever A. F., Love D. R., Robertson J. I. S. and Wilson A.: *Lancet* **11** (1972) 243–247.
5. Melby J. C., Dale S. L. and Wilson T. E.: *Circ. Res.* **28/29** Suppl. 2 (1971) 143–152.
6. Liddle G. W. and Sennett J. A.: *J. steroid Biochem.* **6** (1975) 751–753.
7. Melby J. C. and Dale S. L.: *J. steroid Biochem.* **6** (1975) 761–766.
8. Bullock L. P., Barthe P. L., Mowszowicz I., Orth D. N. and Bardin C. W.: *Endocrinology* **97** (1975) 189–195.
9. Funder J. W., Feldman D. and Edelman I. S.: *Endocrinology* **92** (1973) 944–1004.
10. Marver D., Goodman D. and Edelman I. S.: *Kidney Int.* **1** (1972) 210–223.
11. Suthers M. B., Pressley Lynne and Funder J. W.: *Endocrinology* (submitted).
12. Warburg O. and Christian W.: *Biochem. Z.* **310** (1942) 384–421.
13. De Vries J. R., Ludens J. M. and Fanestil D. D.: *Kidney Int.* **2** (1972) 95–100.
14. Horrobin D. F.: *Prolactin: Physiology and Clinical Significance*, MTP Medical and Technical Publishing Co. Ltd., Lancaster U.K. 1973.
15. Williamson H. E.: *Biochem. Pharmac.* **12** (1963) 1449–1450.
16. Lifschitz M. D., Schrier R. W. and Edelman I. S.: *Am. J. Physiol.* **224** (1973) 376–380.
17. Ehrlich E. N. and Lindheimer M. D.: *J. clin. Invest.* **51** (1972) 1301–1309.