Vol. 124, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1984 Pages 51-56

SULFHYDRYL GROUPS OF ALDOSTERONE RECEPTORS FROM SWINE KIDNEY†

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Received July 31, 1984

SUMMARY Specific [^{3}H]aldosterone binding activity in swine kidney cytosol was inactivated by pretreatment of the cytosol with monoiodoacetamide (pH 8.5), N-ethylmaleimide (pH 7.0), or 5,5'-dithiobis(2-nitrobenzoate) (pH 7.5). Dithiothreitol restored the specific binding activity inactivated by the nitrobenzoate, but not that inactivated with ethylmaleimide. Incubation of the cytosol with aldosterone prior to pretreatment with ethylmaleimide protected the receptors from inactivation. The rank order of steroids for the protection was: aldosterone > hydrocortisone ≥ dexamethazone = progesterone > triamcinolone > estradiol. The initial velocity of the specific hormone binding could be determined by the binding reaction for 60 sec at 30°. Double reciprocal plots of the initial velocity versus the hormone concentration with or without the nitrobenzoate showed a typical pattern of competition between the hormone and the inactivator. The results indicated the presence of functional sulfhydryl groups on the hormone binding sites of aldosterone receptors.

Introduction Aldosterone regulates active sodium reabsorption in mammalian kidneys. This is initiated by the formation of specific aldosterone-receptor complexes (1,2). Although many proposals have been made concerning the mechanism of aldosterone action, only a few reports have described the properties of the aldosterone receptor molecules (1,3). The present study, which was undertaken in order to provide information on the binding site(s) of the receptor, has indicated the presence of sulfhydryl groups in the binding sites of the receptor.

MATERIALS AND METHODS

STEROIDS. [1,2,6,7-^{3}H]Aldosterone (80-100 Ci/m mol) was obtained from Amersham International plc, England. Non-radioactive steroids and spironolactone (17-hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone 7-acetate) were from Sigma Chemical Company.

CYTOSOL PREPARATION. Fresh swine kidneys were obtained from a slaughter house, and their cortices and outer-medullas were stored at -80° until used. All

†This work was supported by grants from Japan Research Foundation for Clinical Pharmacology and Foundation for the Promotion of Research on Medical Resources, Japan.

The abbreviations used are: NEM, N-ethylmaleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoate); and DTT, dithiothreitol.

0006-291X/84 $1.50

subsequent procedures were carried out at 0-5°. Frozen blocks of kidney were homogenized in 1.5 volumes of 10 mM Tris-HCl (pH 7.5) containing 10 mM Na_2WO_4 and 1 mM EDTA with a Polytron homogenizer at half-maximum speed until all large pieces of tissue were disintegrated (approximately 1 min). The homogenate was then centrifuged for 30 min at 100,000xg, and the resultant supernatant (cytosol) was used for the receptor assay.

ASSAY OF ALDOSTERONE RECEPTORS. The quantity of [^{3}H]aldosterone bound to its receptors in the cytosol was determined in a final volume of 0.3 ml of assay medium containing 0.15 ml of cytosol and 5 nM [^{3}H]aldosterone in the presence or absence of nonradioactive aldosterone. Incubation was for 30 min at 20°. Where indicated, the binding was measured at 30° with various incubation periods. After incubation the reaction was stopped by cooling in an ice bath and by the addition of dextran-coated charcoal, consisting of 5% charcoal, 0.5% dextran-T70 (Pharmacia Fine Chemicals), 10 mM Tris-HCl (pH 7.5), 10 mM Na_2WO_4, and 1 mM EDTA. The charcoal was spun down at 3,500 rpm for 10 min, and 0.5 ml of the supernatant was transferred into a scintillation vial. Radioactivity was counted in a scintillation counter at about 37% efficiency following addition of 7 ml of a scintillator mixture consisting of Omniflour and Triton X-100 (4). Specific binding was calculated by subtraction of the binding amount in the presence of excess nonradioactive aldosterone from that in its absence. Protein concentration was determined by the method of Lowry et al.(5).

RESULTS

The aldosterone receptor is extra-labile and loses its hormone binding activity rapidly. Sodium molybdate showed a protective effect on mineralo- and gluco-corticoid receptors of rat kidney (6). Since we observed that sodium tungstate stabilizes aldosterone receptors twice as effectively as molybdate (Tashima, manuscript in preparation), kidney cytosol was prepared in the presence of tungstate. Its final concentration in the binding assay mixture was 2 mM, which was sufficient for the stabilization.

INACTIVATION WITH SULFHYDRYL GROUP REAGENTS. Pretreatment of the cytosol with monoiodoactamide at pH 8.5 or with NEM at pH 7.0 decreased the specific [^{3}H]aldosterone-binding activity more than 80% as shown in Table I. The reaction with the sulfhydryl reagents was stopped by the addition of excess DTT. DTT did not restore the specific binding activity decreased by either monoiodoacetamide or NEM. DTT at the concentrations used showed only a little effect on increasing the specific binding activity in the cytosol without pretreatment: from 6.8 to 7.6 fmol per mg protein (See Table II). The decrease in the specific binding activity by 5 mM NEM reached its maximum within 5 min at 20° (data not shown). This rapid reaction is one of general properties of NEM-modification of functional SH-groups in proteins (7).

Table I. Inactivation of Aldosterone Receptors by Sulfhydryl Group Reagents

Pretreatment with	(mM)	Specific Binding of [^{3}H]Aldosterone (fmol/mg protein)
none		3.9
iodoacetamide	5	0.6
none		3.3
NEM	5	0.3
none		4.0
DTNB	4	0

Pretreatment with iodoacetamide and NEM: swine kidney cytosol (0.2 ml) was mixed with 0.02 ml of 55 mM monoiodoacetamide in 0.5 M Tris-HCl, pH 8.5, or with 55 mM NEM, in 0.5 M Tris-HCl, pH 7.0, or with only the carrier buffer, and incubated for 30 min at 20°. To eliminate the remaining free monoiodoacetamide or NEM, 0.2 M DTT (0.02 ml) was added. The mixture was cooled in an ice bath and assayed for [^{3}H]aldosterone binding as described under MATERIALS AND METHODS. Pretreatment with DTNB: the cytosol (0.2 ml) was mixed with 0.02 ml of 44 mM DTNB in 0.5 M Tris-HCl, pH 7.5, and incubated for 10 min at 20°. Dextran-coated charcoal (0.26 ml) was added to each pretreated mixture, which was then centrifuged. The obtained supernatant was assayed for [^{3}H]aldosterone binding.

Covalent modification of proteins with monoiodoacetamide and NEM is highly specific for SH-groups at pH 8.5 and pH 7.0, respectively, but under certain conditions other groups have been shown to react (7,8). DTNB reacts with SH-groups on proteins, resulting in mixed disulfides. This disulfide-exchange reaction is very specific for SH-groups and is used for measurement of functional SH-groups of proteins (9). The effect of DTNB on the specific binding activity was observed. Since dextran-coated charcoal adsorbs DTNB, a sufficient amount of it was used for stopping the reaction with DTNB. Pretreatment with 4 mM DTNB at pH 7.5 inactivated the specific binding activity completely within 10 min at 20°.

Table II. Reversal of DTNB-Inactivation

Pretreatment with	DTT-treatment	Specific Binding of [^{3}H]Aldosterone (fmol/mg protein)
none	-	6.8
none	+	7.6
DTNB	-	1.5
DTNB	+	6.8

Cytosol (0.2 ml) and 0.02 ml of 0.1 M DTNB in 0.5 M Tris-HCl, pH 7.5, or the carrier buffer were mixed and incubated for 10 min at 20°. Dextran-coated charcoal (0.28 ml) was added, and the tubes were then centrifuged. The supernatant (0.3 ml) was incubated in the presence or absence of DTT (20 mM) for 10 min at 20° and then assayed for the specific binding of [^{3}H]aldosterone.

Table III. Protection from NEM-Inactivation by Steroids

Pretreatment with	(nM)	Remaining Activity (%)
none		8
aldosterone	18	98
hydrocortisone	18	42
dexamethazone	18	39
triamcinolone	18	13
progesterone	18	39
estradiol	900	2
spironolactone	900	63

Cytosol (0.2 ml) and 0.02 ml of nonradioactive steroids were mixed and incubated for 30 min at 20°. After 0.1 M NEM in 0.5 M Tris-HCl, pH 7.0 (0.02 ml) or only the carrier buffer was added to the pretreated mixture, the incubation at 20° was continued for another 7 min. Then 0.2M DTT (0.013 ml) was added to stop the NEM-reaction, and dextran-coated charcoal (0.25 ml) was added. After centrifugation the obtained supernatant was used for assay of specific binding of [^{3}H]aldosterone. Results are shown as percentages of the specific binding of each steroid-pretreated sample without NEM-treatment.

REVERSAL OF THE INACTIVATION WITH DTNB. If DTNB actually inanctivates the specific binding activity by formation of mixed-disulfides on the receptor protein, we might expect that excess DTT would restore the specific binding activity by reduction of the oxidized SH-groups. As shown in Table II, DTT-treatment of the DTNB-pretreated cytosol fully restored the binding activity. The results in Tables I and II indicate the presence of functional SH-groups, whose modification results in loss of specific hormone binding activity.

PROTECTION BY STEROIDS. Incubation of the cytosol with aldosterone prior to the NEM-pretreatment protected the specific binding activity of [^{3}H]aldosterone from inactivation as shown in Table III. Before assaying the binding, free non-radioactive steroids were removed by dextran-coated charcoal, and the exchange binding asssay with [^{3}H]aldosterone was successful. The steroid specificity of the protective effect was examined next. When the cytosol was incubated with 18 nM non-radioactive aldosterone for 30 min at 20° in advance, the inactivation almost completely disappeared. Triamcinolone, highly specific for glucocorticoid receptors and having a negligible affinity to aldosterone receptor, showed little protective effect. The rank order of steroids for protection was aldosterone > hydrocortisone $\geq$ dexamethazone = progesterone > triamcinolone > estradiol, which paralleled to

their binding affinities to the aldosterone receptor (10). Spironolactone, an antagonist of aldosterone (11), binds to the aldosterone receptor with an affinity of 0.1% that of aldosterone (Hama & Tashima, manuscript in preparation). Prior incubation with 900 nM spironolactone protected the receptors from inactivation with NEM partially, 63%. These results suggest the presence of functional SH-groups on the hormone-binding sites of the aldosterone receptor.

COMPETITION BETWEEN DTNB AND ALDOSTERONE. In the concentration range of [^{3}H]aldosterone between 10 to 50 nM, the time course of its specific binding was linear up to 60 sec at 30°. Therefore, the initial velocity of the binding was referred to as the amount of specific binding after a 60 sec incubation. As shown in Fig. 1, the double reciprocal plot of the binding rate versus the hormone concentration with or without DTNB showed the competitive nature of DTNB with respect to specific hormone binding, indicating the presence of specific SH-groups on the hormone binding sites of the aldosterone receptor.

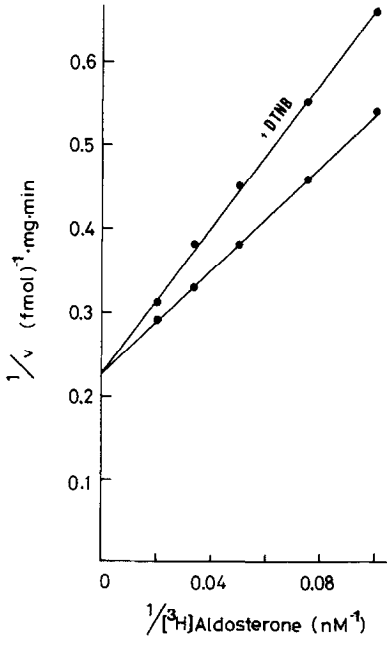

Fig. 1. Effect of DTNB on the rate of specific binding of [^{3}H]aldosterone. The specific binding rate (v) was determined in a final volume of 0.3 ml containing [^{3}H] aldosterone at concentrations from 10 to 50 µM at pH 7.5. Details are described under MATERIALS AND METHODS. The data are expressed as a double-reciprocal plot of the specific binding rate versus the hormone concentration.

DISCUSSION

The present results show the presence of sulfhydryl groups on the hormone-binding sites of aldosterone-receptors. The effects of sulfhydryl group reagents on glucocorticoid receptors have been reported (12,13). Dexamethazone-21-mesylate reacts with sulfhydryl groups extremely fast (14), and binds covalently to glucocorticoid receptors (15). However, neither the specificity nor the kinetic analysis of the mesylate binding have been reported. There is a possibility that the mesylate binding sites are different from the hormone binding sites of the glucocorticoid receptor. Dexamethazone-21-mesylate did not affect the specific aldosterone binding of the swine kidney cytosol (data not shown). In our preliminary experiments, the specific glucocorticoid binding activity in the swine kidney cytosol was also inactivated by NEM and DTNB, and this inactivation was prevented by prior treatment with triamcinolone but not with aldosterone. The presence of SH-groups on the hormone binding sites of both gluco- and mineralo-corticoid receptors suggests the interaction of the SH-groups with the C-20 carbonyl of the gluco- and mineralo-corticoids as discussed by Simons and Thompson (15).

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