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THE BINDING OF CORTISOL TO ADRENAL MITOCHONDRIA

EDUARDO N. COZZA, MARK F. FOECKING and CELSO E. GÓMEZ-SÁNCHEZ*
Department of Internal Medicine, College of Medicine, University of South Florida, and James A Haley VA Medical Center, Tampa, FL 33612, U.S.A.

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Summary—Binding of tritiated cortisol to adrenal zona glomerulosa mitochondria was studied and compared with that of corticosterone. Cortisol was shown to bind specifically to the inner membrane of zona glomerulosa mitochondria. Corticosterone and cortisol had similar apparent association constants (K_a) and concentrations of binding sites. The methodology was validated by obtaining similar K_a from both binding plots and kinetic data. Cortisol binding was inhibited by pretreatment with sodium dithionite, and displaced by deoxycorticosterone, corticosterone, 18-hydroxy-corticosterone, 11β-hydroxy-18-ethynil-progesterone and metyrapone, but not by cholesterol. These results suggest that cortisol and corticosterone bind to the same cytochrome *P*-450.

INTRODUCTION

The isolation and identification of 18-hydroxycortisol (18-OH-F) from the urine of patients with primary aldosteronism was recently described by Chu and Ulick [1]. Patients with primary aldosteronism also excrete excessive amounts of another 18-hydroxylated steroid, 18-oxo-cortisol (18-oxo-F) [2, 3]. Cortisol (F) can be metabolized by bullfrog interrenal organ to 18-OH-F and 18-oxo-F probably using the same pathway by which corticosterone is converted to 18-hydroxy-corticosterone (18-OH-B) [1, 4].

Adrenal cells originate in the subcapsular region of zona glomerulosa and produce the 17-deoxysteroids, corticosterone, 18-OH-B and aldosterone. As cells migrate centripetally, the most important enzyme in the synthesis of aldosterone, the cytochrome *P*-450 corticosterone methyl oxidase [5], diminishes its activity and the cytochrome *P*-450 17-hydroxylase is induced under the effect of ACTH [6, 7]. We have previously postulated that in the interphase between the zona glomerulosa and zona fasciculata, there is an area with morphological and functional characteristics which share features of both the zona glomerulosa and fasciculata which we have called transitional zone [8]. In this area, the cytochrome *P*-450 corticosterone methyl oxidase is exposed to cortisol, postulated to be a suboptimal substrate, resulting in the synthesis of the 17-hydroxylated analogs of 18-OH-B and aldosterone, 18-OH-F and 18-oxo-F, respectively [1]. The first step in the metabolic transformation of corticosterone by the mitochondrial cytochrome *P*-450 involves binding to the enzyme. We are herein reporting results of binding studies of corticosterone and cortisol to bovine adrenal mitochondria.

*To whom all correspondence should be addressed: Dr Celso E. Gómez-Sánchez, Department of Medicine, James A Haley VA Hospital, 13000 Bruce B Downs Blvd-VAH 111M, Tampa, FL 33612, U.S.A. [Tel. (813)-972-7624; *Fax* (813)-972-7623].

MATERIALS AND METHODS

Steroids were obtained from Sigma Chemical Company (St Louis, Mo.). [1,2,6,7-^{3}H]corticosterone and [1,2,6,7-^{3}H]cortisol were purchased from Amersham Corporation (Arlington Heights, Ill.). 11β-Hydroxy-18-ethynil-progesterone was generously provided by Dr J. O. Johnston from Merrell Dow Research Laboratories. Freshly frozen beef adrenal glands were obtained from Pel-Freez (Rodgers, Ariz.). Fresh calf adrenals were obtained from a local abattoir, transported immediately to the laboratory in ice-cold Hank's balanced salt solution.

Isolation of outer adrenal cell mitochondria

Frozen bovine adrenals were carefully thawed in the refrigerator and trimmed of fat. A 500 μ outer layer was sliced off using a Stadie-Riggs Microtome. In some experiments fresh calf adrenals were used to validate the results obtained with frozen adrenals. The slices were minced and homogenized in ice-cold homogenization buffer (sucrose 0.25 M, Hepes 20 mM, pH 7.1) using a Polytron Homogenizer (Brinkman Instruments, New York). The homogenate was then centrifuged at 600 *g* × 10 min. The pellet was resuspended and centrifuged again. The combined supernatants were centrifuged at 12,000 *g* × 20 min. The pellet was resuspended again in the same buffer and recentrifuged. The final pellet was suspended in binding buffer (Hepes 20 mM,

sucrose 0.025 mM, 0.2% bovine serum albumin, 10 μg/ml leupeptin and 0.1 mM PMSF).

Reference enzymes

Succinate dehydrogenase [9], glucose-6-phosphatase [10], glucose-6-phosphate dehydrogenase [11] and kynurenine hydroxylase [12], and DNA [13] were measured to check the purity of subcellular fractions.

Mitochondria fractionation

Mitochondria were broken using hypo-osmotic shock [14] separated into two fractions, the mitoplast, which is composed of inner membrane plus matrix, and the outer mitochondrial fraction, composed of outer mitochondrial membrane and interspace components.

The outer fraction had 97% of total kynurenine hydroxylase activity, an outer mitochondrial membrane marker, and no succinate dehydrogenase activity, an inner mitochondrial membrane marker. Mitoplasts showed 100% of total succinate dehydrogenase activity and 4% of total kynureine hydroxylase activity. These results indicate that the outer mitochondrial fraction obtained was pure and that the mitoplasts were very slightly contaminated with outer membrane components.

Binding experiments

Steroid binding to mitochondria was performed essentially as described by Satre and Vignais [15]. The steroids dissolved in ethanol were added to 12 × 75 polypropylene tubes and evaporated under a gentle stream of air at room temperature and redissolved in 15 μl of propyleneglycol. The mitochondria in 1 ml of binding buffer containing 3–3.5 mg of protein were added to pre-cooled tubes and incubated at 0°C for 10 min. The bound steroid was separated from unbound by centrifugation at 25,000 *g* × 15 min and radioactivity was measured in the supernatant. Non-specific binding was determined in parallel incubations with 100-fold excess unlabeled steroid.

Similar experiments were also performed using nuclei, mitoplasts, cytosol and outer mitochondria fraction. For the latter two soluble fractions, free steroid were adsorbed using 1 ml of 0.15% Norit-A charcoal, 0.015% dextran T-70 in 0.05 M borate buffer, pH 8, and centrifuged at 5000 *g* × 30 min. The supernatant was decanted into vials and counted. Radioactivity was measured using a LKB Liquid Scintillation Counter.

Dissociation constants and concentration of binding sites were calculated using the Lineweaver–Burk plots (1/[bound steroid] vs 1/[free steroid]).

RESULTS

Time-course of binding to mitochondria

Mitochondria were incubated at 0 and 37°C for various periods of time. The short incubations were accomplished by incubating the tubes in a cooled centrifuge rotor and centrifuged for the time stated. Maximum binding was obtained between 5 and 30 min. Binding periods greater than 30 min showed more variance, specially at the higher temperature. A 10 min incubation time was selected for the binding studies.

Table 1. Subcellular distribution of cortisol binding to bovine adrenal zona glomerulosa

		Nuclei	Mitochondria
		Subcellular markers	
%DNA		96.2	2.6
%SDH		1.3	98.4
%Glu-Pase		1.3	1.8
%Glu-6-P-Dase		1.4	0.0
		Ligands	
B	K_d (nM)	7.91 ± 0.93	10.72 ± 1.32
	B_{max} (nM/mg prot.)	0.032 ± 0.006	0.575 ± 0.071
	n	3	7
F	K_d (nM)	ND	13.44 ± 1.98
	B_{max} (nM/mg prot.)	ND	0.52 ± 0.06
	n	ND	5

Data of subcellular markers are representative of one experiment. Data of binding are mean ± SEM of the number of experiments shown. K_d and B_{max} were calculated from Lineweaver–Burk plots for which 7 different concentrations of tritiated cortisol, in triplicate, were used in each experiment. SDH, succinate dehydrogenase; Glu-6-Pase, glucose-6-phosphatase; Glu-6-P-Dase, glucose-6-phosphate dehydrogenase; B, corticosterone; F, cortisol; K_d, dissociation constant; B_{max}, concentration of binding sites; *n*, number of independent experiments.

Subcellular, zonal and submitochondrial binding

Nuclei, mitochondria and soluble fractions from whole bovine adrenals were used for binding experiments with tritiated cortisol. The soluble fraction did not show specific binding. The apparent K_d, concentration of binding sites and purity of the fractions are shown in Table 1. The nuclear fraction had some binding which can be explained by mitochondrial contamination. A Lineweaver–Burk plot of the binding of tritiated cortisol to adrenal mitochondria is shown in Fig. 1. Binding of tritiated corticosterone to mitochondria was similar to that of cortisol (Table 1).

Mitochondria from zona glomerulosa bound cortisol with K_d of 9.96 ± 0.94 nM (mean ± SEM, $n = 4$) and the concentration of binding sites was 1.39 ± 0.10 nM/mg protein (mean ± SEM, $n = 4$). This K_d is similar to the one obtained from whole adrenals but the concentration of binding sites is two

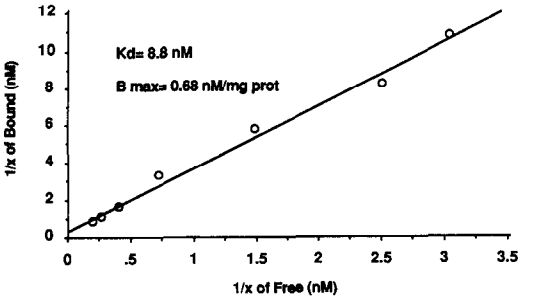

Fig. 1. Lineweaver–Burk plot of the binding of tritiated cortisol to adrenal mitochondria. Each point is the mean of triplicates. SEM bars are smaller than sympol heights.

Table 2. Characteristics of cortisol binding to mitochondrial fractions of bovine adrenal zona glomerulosa

Fraction	K_d (nM)	B_{max} (nM/mg prot.)
Mitoplasts	12.71 ± 1.84	0.84 ± 0.11
Sonicated mitochondria	9.71 ± 1.12	0.59 ± 0.08
Pellet from sonicated mitochondria	13.47 ± 1.91	0.92 ± 0.10

Data are mean ± SEM of 3 different experiments. K_d and B_{max} were calculated from Lineweaver–Burk plot for which 7 different concentration of tritiated cortisol, in triplicate, were used in each experiment.

times higher in zona glomerulosa than in total adrenal mitochondria. This suggests that most, if not all, of the binding occurs in the zona glomerulosa.

In order to confirm this hypothesis, cortisol binding was also measured in the second 500 μ-layer of the adrenal. These experiments showed that while K_a was similar in both layers (8.73 ± 0.77, mean ± SEM, $n = 3$), the concentration of binding sites was lower in the second adrenal layer (0.42 ± 0.04, mean ± SEM, $n = 3$) than in the first layer.

Experiments using whole adrenal mitochondria and zona glomerulosa mitochondria were repeated using fresh calf adrenals, instead of fresh frozen bovine adrenals. Results for both adrenal sources were indistinguishable.

No significant differences were found in K_ds and concentration of binding sites between whole mitochondria, sonicated mitochondria or 17,000 g × 20 min-pellets of sonicated mitochondria and mitoplasts (Table 2). No specific binding was found either with the outer mitochondria fraction or with the 17,000 g × 20 min-supernatant of sonicated mitochondria. These results suggest that the binding site for cortisol is localized in the inner mitochondrial membrane.

Temperature dependency

Mitochondria were incubated as above for 10 min at 0, 10, 20 and 30°C and the apparent K_d obtained. The different K_d were plotted against 1/T (K), Fig. 2, from which the enthalpy change, $\Delta H = -2.909$ kcal/mol, can be derived from the slope, and the entropy change, $\Delta S = 25.81$ kcal/mol · K, can be derived from the ordinate. Table 3 shows the entropy (S) and Gibbs energy (G) changes involved in binding of cortisol to mitochondria at all temperatures used, as well as the enthalpy (H) change between two consecutive temperatures. From these results, it can be seen that the binding of cortisol to mitochondria is

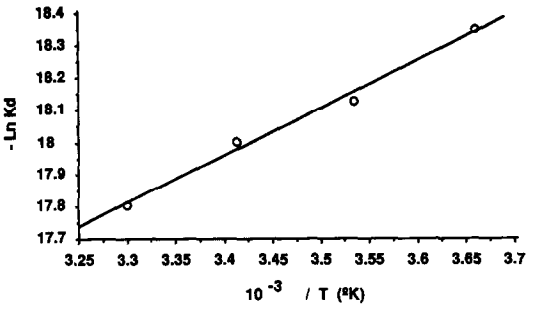

Fig. 2. Temperature dependency of K_d for the binding of cortisol to adrenal mitochondria. K_ds at each temperature were calculated from Lineweaver–Burk plot, which were performed using 7 different concentrations of tritiated cortisol.

exothermic, spontaneous and occurs with an increase in entropy.

Association and dissociation specific constants

The specific association constant (k_1) was obtained by doing a time-course to 3 min. Figure 3 shows the plot from which k_1 can be derived, resulting $1.126 \times 10^5\ M^{-1}\ sec^{-1}$. The specific dissociation constant (k_2) was obtained after adding 1 μmol of unlabeled cortisol in 10 μl of ethanol to mitochondria preincubated for 10 min with tritiated cortisol. The specific dissociation constant was $1.34 \times 10^{-3}\ sec^{-1}$. K_d is calculated from these kinetic data as k_2/k_1, obtaining $K_d = 11.9$ nM which is very close to the mean determined by other experiments (Table 1).

Effect of sodium dithionite

Reduction of the cytochrome P-450 by 5 min pretreatment with sodium dithionite strongly decreased the binding of cortisol to zona glomerulosa mitochondria. In effect, K_d was increased from 10.24 to 64 nM, and the concentration of binding sites was decreased from 1.46 to 0.39 nM/mg protein (mean, $n = 3$).

Displacement

Tritiated cortisol was incubated with zona glomerulosa mitochondria in the presence of 100-fold excess of different unlabeled steroids and metyrapone. Binding of tritiated cortisol was displaced by cortisol (100%), deoxycorticosterone (84%), corticosterone (79%), 18-OH-F (24%), 18-OH-B (34%), metyrapone (96%) and 11β-OH-18-ethynil-progesterone (100%), but not by cholesterol (6%).

Table 3. Thermodynamic parameters of the binding of cortisol to bovine adrenal zona glomerulosa mitochondria

T(K)	n	K_d (nM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (kcal/mol · K)
273	7	10.72	−9.95		25.80
283	3	13.42	−10.19	−3.46	25.73
293	3	15.18	−10.48	−2.02	25.84
303	3	18.56	−10.72	−3.53	25.77

K_ds were calculated from Lineweaver–Burk plots. ΔG, ΔH and ΔS were calculated as described by Satre and Vignais [15].

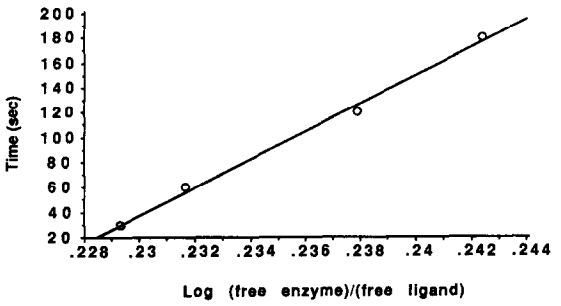

Fig. 3. Determination of the specific association constant (k_1) for the binding of cortisol to adrenal mitochondria. This plot was derived from the time-course of the association of cortisol to adrenal zona glomerulosa mitochondria according to [15]. In parallel, B_{max} was calculated from Lineweaver–Burk plots to obtain the concentration of free protein by subtracting [bound] from B_{max}.

11β-OH-18-Ethynil-progesterone is a time-dependent inhibitor of the production of aldosterone by adrenal zona glomerulosa mitochondria.

DISCUSSION

The present work shows that cortisol binds to the inner mitochondrial membrane of adrenal zona glomerulosa. In effect, binding of cortisol was localized in the mitoplast and in the pellet, but not in the supernatant of sonicated mitoplast (not shown) or mitochondria (Tables 1 and 2).

The thermodynamic studies of such binding proved to be exothermic and spontaneous and producing an increase in entropy. These conditions are very similar to those previously reported for metyrapol [15]. The similar results obtained in calculating K_d using kinetic data and the Lineweaver–Burk plot, validate of the methods used in this study.

The submitochondrial localization of the cortisol binding in the inner membrane and the fact that sodium dithionite pretreatment inhibited the binding of cortisol to adrenal mitochondria suggests that cortisol is bound to a mitochondrial cytochrome *P*-450.

The displacement results indicate that deoxycorticosterone, corticosterone, 18-OH-B, 11β-OH-18-ethynil-progesterone and metyrapone, all related to the late pathway of aldosterone biosynthesis, displace the binding of cortisol to adrenal zona glomerulosa mitochondria. Cholesterol was ineffective in displacing cortisol. These findings are in agreement with a previous report in which it is suggested that cortisol is metabolized to 18-OH-F and 18-oxo-F using the same pathway by which corticosterone is converted into 18-OH-B and aldosterone. The fact that the concentration of binding sites for both corticosterone and cortisol are the same (Table 1), also supports these ideas.

Finally, our findings suggest that cortisol and corticosterone bind to the same site in adrenal zona glomerulosa mitochondria, and that this binding site is a cytochrome *P*-450. These results support the idea that cortisol may be metabolized through the same pathway by which corticosterone is converted into aldosterone. The relationship between this pathway, the putative transitional zone and primary aldosteronism may be relevant to the understanding of this complicated clinical syndrome.

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