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Inhibition of aldosterone formation by cortisol in rat adrenal mitochondria

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In this work we confirm by a metabolic method the existence of at least two enzymes with 11β- and 18-hydroxylase activities in rat adrenal mitochondria. The method was based on the ability of cortisol (F), a foreign alternative substrate, to inhibit competitively metabolite productions from various precursors. F inhibited a) aldosterone (ALDO) production from 11-deoxycorticosterone (DOC) without affecting the yields of corticosterone (B) and 18-hydroxy-11-deoxycorticosterone (18-OHDOC); b) 18-hydroxycorticosterone and aldosterone productions from B (K_i = 2.5 ± 0.5 μM); and c) ALDO production from 18-OHDOC. These results suggest the existence of two categories of enzymes with both 11β- and 18-hydroxylase activities, one comprising those that catalyze the conversions of DOC to B and 18-OHDOC (F-insensitive reactions [FIS]) and the other one comprising the enzymes involved in the conversions of B to 18-OHB and ALDO and that of 18-OHDOC to ALDO (F-sensitive reactions [FS]). The cloned enzymes CYP11B1 and CYP11B2 would pertain respectively to the FIS and FS categories. (Steroids **60**:447–452, 1995)

Keywords: aldosterone; adrenal; cytochrome P450; 11β, 18-hydroxylases; steroidogenesis; Cortisol

Introduction

The main biosynthetic pathway from 11-deoxycorticosterone to aldosterone in rat adrenals involves the following sequence: 11-deoxycorticosterone—corticosterone—18-hydroxycorticosterone—aldosterone.[1–2] These conversions, consisting of hydroxylations at positions 11β or 18, are NADPH- and O_2-dependent and are catalyzed by enzymes of the cytochrome P450 family.[2–3]

Human, mouse, and rat adrenals possess two cytochrome P450 enzymes[4–8] with 11β-hydroxylase activity, each of which also has 18-hydroxylase activity, although this latter activity is substrate-dependent for each enzyme. Thus, both enzymes catalize 11-deoxycorticosterone to corticosterone, but only CYP11B1 catalyzes 11-deoxycorticosterone to 18-hydroxy-11-deoxycorticosterone,[9–12] while only CYP11B2 catalyzes corticosterone to 18-hydroxycorticosterone and aldosterone.[12] All these biosynthetic pathways were deduced by using purified enzymes. No validation has been reported with mitochondria as enzyme sources. Such a validation, however, is important, since the activities of adrenal steroidogenic cytochromes P450 are extremely dependent on their lipid environment,[13–15] the presence of endogenous antioxidants[16–17] and dilution[18] among other factors.

It is well accepted that CYP11B2 is confined to zona glomerulosa and that CYP11B1 exists in zona fasciculata. The existence of CYP11B1 in zona glomerulosa is still under discussion. Several groups support[19–21] and others reject[22–24] such a possibility.

18-Hydroxycorticosterone is produced by the three corticoadrenal zones but aldosterone is only formed by glomerulosa. No clear explanation exists for the exclusive biosynthesis of aldosterone in this zone.[2]

Cortisol is not produced by rat adrenals. In bovine adrenal preparations it is converted into 18-hydroxycortisol and 18-oxocortisol[25–27] using the same enzymatic systems as corticosterone in its transformation to 18-hydroxycorticosterone and aldosterone. Emeric-Blanchouin et al.[28] confirmed that cortisol inhibited the conversions of both corti-

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costerone and 18-hydroxycorticosterone into aldosterone by duck adrenals.

As will be shown in the Discussion, such a foreign inhibitor is useful for the categorization and characterization of enzymes present in a given tissue.

In this report, we use cortisol to inhibit the formation of aldosterone from different substrates and to categorize the reactions involved in the metabolism of 11-deoxycorticosterone by the adrenal cortex into cortisol-sensitive and cortisol-insensitive enzyme reactions.

Experimental

Chemicals

[1,2-^{3}H]11-deoxycorticosterone, [1,2-^{3}H]corticosterone and [1,2-^{3}H]18-hydroxycorticosterone were purchased from the Amersham Corporation (Arlington Heights, IL, USA). Radioactive and radioinert 11,18-epoxycorticosterone (18-deoxyaldosterone[29–31]) were obtained from 18-hydroxycorticosterone by the *p*-toluensulfonic acid method.[25] Other radioinert steroids and reagents for buffers were from Sigma Chemicals (St. Louis, MO, USA).

Preparation and incubation of mitochondria

Male CHBB-Thom rats (150–200 g) were sacrificed by decapitation and adrenals were immediately removed, placed in ice-cold saline, trimmed free of fat and surrounding tissue, and finally decapsulated. Adrenal capsules were homogenized in Krebs-Ringer-bicarbonate-glucose buffer[31] (pH = 7.2), and mitochondria were separated by centrifugation as reported elsewhere.[32] The final mitochondrial pellet was reconstituted in the same buffer and aliquots (0.6 mg mitochondrial protein) were incubated in a final volume of 0.5 mL in the presence of radioactive steroids as substrate, with or without other radioinert steroids (see Results). Incubations were started by the addition of 0.5 mM NADPH and stopped by placing tubes into an ice-water bath for 15 min. Incubation supernatants were obtained by centrifugation of samples at 2500 rpm for 10 min and extracted twice with 5 vol methylene chloride. The recovery of this extraction was very constant among samples and averaged 96%.

Separation and quantification

Customarily, traces of labeled steroid are added to a system containing unlabeled steroid to estimate efficiency of recovery.[33] By contrast, we used radioinert steroids added to radioactive steroids (organic extracts) to determine recoveries after TLC separation and HPLC quantification. This last procedure has been successfully employed by us[27] and is highly recommended as an alternative choice in biosynthetic studies.

After adding a known amount (6 μg) of the radioinert steroid to be measured, organic extracts were spotted on silica gel plates and chromatographed with methylene chloride:methanol:acetonitrile (86:7:7, v/v/v) as a mobile phase. Position of products was revealed by UV light, and silica corresponding to each steroid was carefully scraped off and used as packing of small columns. For this purpose, silica (80 to 120 mg depending on the steroid spot) was packed into 1 cm × 5 cm conic plastic columns (height of silica 1 cm). Steroids were then eluted from these small columns with methylene chloride:methanol (95:5, v/v). Eluates were evaporated under N_2 at 37°C, reconstituted with MeOH, and resolved using HPLC (P-100 pump, UV-100 detector, and SP-4600 integrator from Spectra Physics Analytical [Fremont, CA, USA] and 7125 NS injector from Rheodyne [Cotati, CA, USA]). Conditions for HPLC chromatography were as follows: column, μ-Bondapack C-18; detection, absorbance at 254 nm; mobile phase, methanol:water (3:2, v/v); flow rate, 0.8 mL/min.

Effluent from the HPLC column was collected in 0.26 mL fractions and radioactivity measured by liquid scintillation counting. The radioactivity of the steroid corresponding to each peak elicited in the HPLC chromatogram (hereafter referred to as peak-radioactivity) was calculated by summing the radioactivity of the collected fractions associated to the peak.

The mass of the steroid corresponding to the peak elicited in the HPLC run (hereafter referred to as peak-mass) was calculated by comparison with a calibration curve carried out with standards.

Recoveries were estimated by the following formula: percent recovery = peak-mass × Vt × 100/(Va × 6 μg) where Vt is the total volume of the sample analyzed by HPLC, and Va is the volume of the aliquot injected into the HPLC equipment (25 μL). Since the masses of endogenous steroids are negligible in comparison to the mass of the exogenous steroid added to the organic extracts (6 μg), the calculated peak-mass may be taken as corresponding to this exogenous tracer.

Recoveries were then applied to peak-radioactivity using: total radioactivity of steroid X = peak-radioactivity × 100/(percent recovery), where steroid X is the steroid to be determined.

Usual retention times obtained were: 4.9 min for corticosterone, 6.9 min for aldosterone, and 7.8 min for 18-hydroxycorticosterone.

Purity of the radioactive steroids collected after HPLC was confirmed through crystallization to constant specific activity. For this purpose, to the organic extract of the incubation medium was added 6.3 mg of unlabeled aldosterone which was then recrystallyzed to constant specific activity (SA_1). On the other hand, to the aldosterone-like radioactive material eluted from HPLC was added an amount of unlabeled aldosterone equal to (6.3 mg × percent recovery/100). The specific activity of this fraction after recrystallization (SA_2) was compared to SA_1. The criteria used to confirm purity of the steroid collected after HPLC was as follows: 1) SA_2 lower than SA_1 would indicate no purity, 2) SA_2 equal to SA_1 would indicate purity, and 3) SA_1 lower than SA_2 would indicate a mistake, since this condition is senseless.

In a few experiments, tritiated cortisol was used as substrate and radiometabolites were analyzed as before.

Proteins were measured by the method of Bradford.[34] Specific binding of cortisol and 18-hydroxycortisol to adrenal mitochondria was determined as already reported.[32]

Results

Quantification of radiometabolites

In preliminary studies, we determined the purity of steroids isolated from incubation media after TLC-HPLC separation (see Experimental) by measuring the specific activity of the radioactive material after recrystallization in acetone/water.[35,36] To the organic extract of the incubation medium was added 6.3 mg of unlabeled aldosterone which were then recrystallized.

The specific activity decreased after subsequent recrystallizations from 333.0 dpm/μg until reaching a constant value of 91.6 dpm/μg (SA_1).

On the other hand, to the aldosterone-like radioactive material eluted from HPLC (see Experimental) was added an amount of unlabeled aldosterone equal to the product 6.3 mg × percent recovery/100 after HPLC (see Experimental).

The specific activity of this fraction was 92.0 dpm/μg (SA_2). Subsequent recrystallization of this fraction did not decrease this value (91.6 dpm/μg).

The similarity between SA_1 and SA_2 allows us to conclude that aldosterone-like material after TLC-HPLC separation is almost exclusively aldosterone (See experimental). Similar results were obtained for other products.

Biosynthetic capacity for aldosterone

The formation of aldosterone from 1 μM 11-deoxycorticosterone was linear up to 45 min (18 ± 2, 35 ± 3, and 54 ± 5 ng aldosterone/tube for 15, 30, and 45 min incubation time, respectively; mean ± range of two experiments performed in duplicates.

The formations of 18-hydroxy-11-deoxycorticosterone, corticosterone, and aldosterone from 11-deoxycorticosterone, as well as of 18-hydroxycorticosterone and aldosterone from corticosterone, were measured at different substrate concentrations. The apparent K_M and V_{max} were obtained by Lineweaver-Burk plots and are shown in Table 1.

Effects of cortisol

The addition of 10 μM cortisol to incubation with 1 μM 11-deoxycorticosterone decreased the production of aldosterone while the productions of 18-hydroxy-11-deoxycorticosterone and corticosterone were not affected or slightly increased (Table 2). These results suggest that cortisol is decreasing the production of aldosterone from 11-deoxycorticosterone by inhibiting a step beyond corticosterone formation.

Additionally, the presence of 10 μM cortisol also decreased the production of 18-hydroxy-corticosterone and aldosterone from corticosterone (Table 2), thus confirming that cortisol inhibits corticosterone metabolism.

In order to investigate the type of inhibition cortisol exerts on aldosterone production from corticosterone, Lineweaver-Burk plots were obtained. Figure 1 shows a typical competitive plot for the cortisol-mediated inhibition of aldosterone formation from corticosterone, with a K_i of 2.5 ± 0.5 μM (mean ± SEM).

Binding and metabolism of cortisol

We have previously shown in bovine adrenal zona glomerulosa that cortisol and corticosterone share binding sites to the oxidized state of a mitochondrial cytochrome P450, cortisol thus demonstrating to be itself a competitive inhibitor of the transformation of corticosterone into aldosterone.

To test this hypothesis for rat adrenal mitochondria, we performed binding studies with this preparation.

Tritiated cortisol specifically binds to rat adrenal zona glomerulosa mitochondria. This binding was displaced by radioinert cortisol in a dose-dependent manner (Table 3). The addition of corticosterone and 18-hydroxy-11-deoxycorticosterone, but not of 11-deoxycorticosterone, also significantly decreased the binding of cortisol (Table 3).

When 1 μM tritiated cortisol was incubated with rat adrenal mitochondria in the presence of 0.5 mM NADPH for 40 min, and the radiometabolites analyzed by thin-layer chromatography (see Experimental), the main peak of radioactivity comigrated with standard [^{3}H]18-hydroxycortisol. The percentage of conversion was 4.0 ± 1.2% (mean ± range, n = 2).

Transformation of 11,18-epoxycorticosterone into aldosterone

11,18-Epoxycorticosterone was converted into aldosterone by our mitochondrial preparation (Table 1).

The addition of 10 μM cortisol decreased this conversion (Table 2). The Lineweaver-Burk plot for the conversion of 11,18-epoxycorticosterone into aldosterone in the presence of cortisol showed a competitive effect of this last steroid (Figure 1).

The conversion of 11,18-epoxycorticosterone into aldosterone was also decreased by 41 ± 1% ($P < 0.02$, analysis of variance) in the presence of 1 μM 11-deoxycorticosterone.

On the other hand, 11,18-epoxycorticosterone inhibited

Table 1 Apparent K_M and V_{max} (mean ± SEM)

Substrate	Product	K_M (μM)	V_{max} (nmol/min × mg protein)
DOC	B	17.9 ± 2.1	16.5 ± 2.9
DOC	18 OHDOC	36.3 ± 4.9	53.2 ± 4.2
DOC	ALDO	22.5 ± 3.7	1.27 ± 0.08
B	18 OHB	3.2 ± 0.6	1.37 ± 0.09
B	ALDO	1.3 ± 0.2	0.69 ± 0.09
18 DAL	ALDO	4.7 ± 0.9	0.11 ± 0.02

Abbreviations used: DOC,11-deoxycorticosterone; B, corticosterone; 18 OHDOC, 18-hydroxy-11-deoxycorticosterone; ALDO, aldosterone; 18 DAL, 11,18-epoxycorticosterone; 18OHB, 18-hydroxycorticosterone.
K_M and V_{max} values were calculated from Lineweaver-Burk plots which were obtained with six different substrate concentrations in duplicates.

Table 2 Effects of cortisol

Substrate (1 μM)	Product	Cortisol (10 μM)	Rate
DOC	B	−	1.00 ± 0.05
		+	1.22 ± 0.11[a]
DOC	18 OHDOC	−	1.00 ± 0.09
		+	1.18 ± 0.11
DOC	ALDO	−	1.00 ± 0.03
		+	0.40 ± 0.06[b]
B	18 OHB	−	1.00 ± 0.15
		+	0.21 ± 0.03[a]
B	ALDO	−	1.00 ± 0.06
		+	0.43 ± 0.07[a]
18 OHDOC	ALDO	−	1.00 ± 0.15
		+	0.45 ± 0.06[c]
18 OHB	ALDO	−	1.00 ± 0.16
		+	0.69 ± 0.03
18 DAL	ALDO	−	1.00 ± 0.05
		+	0.35 ± 0.05[b]

Rate: Transformation rate of substrates into the indicated products in the presence (+) of cortisol/same transformation rate in the absence (−) of cortisol. For abbreviations see Table 1.
Results are mean ± SEM of 2 to 4 experiments performed in duplicate.
[a] $P < 0.02$. [b] $P < 0.01$. [c] $P < 0.05$.
All P values with respect to control with no cortisol Analysis of variance (ANOVA).

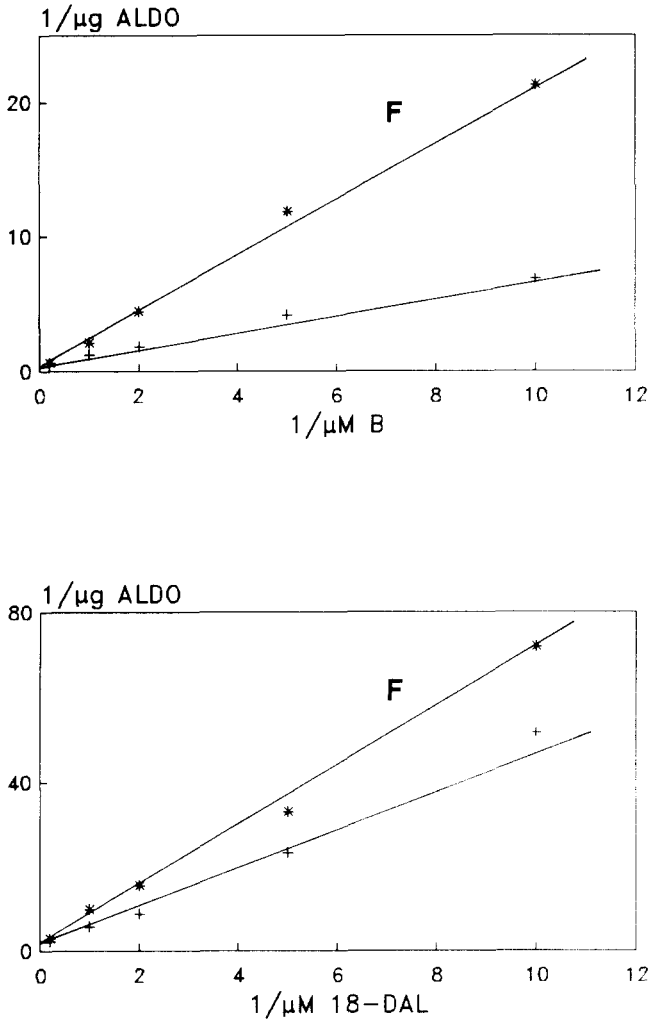

Figure 1 Lineweaver-Burk plots for the formation of aldosterone from corticosterone (B; upper panel) and 11,18-epoxycorticosterone (18 DAL; lower panel) in the absence (+) or presence (*) of 10 µM cortisol (F).

the conversions of 11-deoxycorticosterone and 18-hydroxycorticosterone into aldosterone but not that of 11-deoxycorticosterone into corticosterone and 18-hydroxy-11-deoxycorticosterone (Table 4). Thus 11,18-epoxycorticosterone has an inhibitory pattern similar to cortisol on the last steps of aldosterone biosynthesis.

Discussion

Foreign alternative substrates are substances that are not produced by the tissue but bind to and compete for the same enzyme sites as endogenous intermediates. In other words, foreign alternative substrates inhibit the conversion of radioactive intermediates to end-products by competing for common enzymes, thereby accumulating radioactivity in those intermediates and decreasing it in end-products.

Cortisol fulfills the conditions for a foreign competitor described above. Indeed: a) it is not produced by rat adrenals which notoriously lack 17-hydroxylase activity; b) publications of the last two decades first suggest[25] then confirm[27] that F is transformed in adrenals of other species into

Table 3 Specific binding of cortisol to rat adrenal

Unlabeled cortisol (µM)	Other unlabeled steroids (10 µM)	Specific binding (cpm)
0	—	5340 ± 287
0.01	—	5224 ± 365
0.1	—	4238 ± 207
1	—	1540 ± 87[a]
10	—	389 ± 38[a]
100	—	62 ± 9[a]
—	B	224 ± 18[a]
—	18 OHDOC	376 ± 26[a]
—	DOC	4779 ± 215

Data are mean ± SEM of three experiments performed in duplicates. —, No addition.
For abbreviations see Table 1.
[a] $P < 0.01$ with respect to control with no unlabeled steroids (ANOVA).

18-hydroxycortisol and 18-oxocortisol by the enzyme that converts corticosterone to aldosterone. The first results were obtained by Chu and Ulick, who isolated 18-oxocortisol from urine of patients with primary aldosteronism.[37] Furthermore, 18-oxocortisol and 18-hydroxycortisol are secreted in excess by patients with adrenal adenomas and glucocorticoid-supressible aldosteronism.[38]

More recently, kinetic evidence was obtained that cortisol and corticosterone compete for a common cytochrome P450 in duck adrenal mitochondria[28] and bovine glomerulosa mitochondria.[27] The latter work indicates that cortisol is metabolized to 18-hydroxycortisol and 18-oxocortisol by a mitochondrial cytochrome P450 identical to that catalyzing the conversion of corticosterone to aldosterone. Moreover, in bovine glomerulosa, corticosterone and cortisol bind to the same mitochondrial proteins,[32] a finding confirmed for rat adrenal mitochondria in the present work (Table 3).

In this study we analyze the effects of the foreign alternative substrate cortisol on the aforementioned enzymic steps from 11-deoxycorticosterone to corticosterone, 18-hydroxycorticosterone, and aldosterone, as well as other steps between 11-deoxycorticosterone and aldosterone in rat adrenals.

Table 4 Effects of 11,18-epoxycorticosterone (18 DAL)

Substrate (1 µM)	Product	18 DAL (10 µM)	Rate
DOC	B	—	1.00 ± 0.09
		+	1.09 ± 0.08
DOC	18 OHDOC	–	1.00 ± 0.12
		+	1.04 ± 0.06
DOC	ALDO	–	1.00 ± 0.08
		+	0.45 ± 0.05[a]
18 OHB	ALDO	–	1.00 ± 0.06
		+	0.57 ± 0.07[b]

Rate: Transformation rate of substrate into the indicated products in the presence (+) of 18 DAL/same transformation rate in the absence (–) of cortisol. For abbreviations see Table 1.
Results are mean ± SEM of 2 to 4 experiments performed in duplicates. [b] $P < 0.05$, [a] $P < 0.02$, with respect to controls with no 18 DAL (ANOVA).

The steps inhibited by cortisol were the transformations of corticosterone to 18-hydroxycorticosterone, 18-hydroxycorticosterone to aldosterone, 18-hydroxy-11-deoxycorticosterone to aldosterone, and 11,18-epoxycorticosterone to aldosterone. These inhibitions showed to be competitive, according to Lineweaver-Burk plots and binding experiments. On the other hand, no inhibition by cortisol was obtained in the conversion of 11-deoxycorticosterone to corticosterone and 18-hydroxy-11-deoxycorticosterone.

As mentioned in the Introduction, two cytochromes P450 have been isolated and cloned from rat adrenals; one, CYP11B1, catalyzes the reactions of 11-deoxycorticosterone to corticosterone and 18-hydroxy-11-deoxycorticosterone occurring in zona fasciculata. The second one, CYP11B2, only present in zona glomerulosa, catalyzes these reactions and also possesses methyloxidase I and II activities (i.e., the activities of "18 hydroxylase of corticosterone" and "aldo synthase").[4–12]

At variance with the zonation of CYP11B2, that of CYP11B1 is still a matter of considerable controversy. While some publications maintain that CYP11B1 is only present in fasciculata-reticularis,[22–24] others consider the presence of this enzyme in fasciculata-reticularis and glomerulosa.[19–21] Our results may be due either to the validity of this second point of view or to contamination of our preparation with mitochondria from fasciculata-reticularis. This last assumption seems plausible, since the 11β-hydroxylating activity of CYP11B1 is much stronger than that of CYP11B2. (Hence, even a small contamination of our preparation with fasciculata mitochondria could lead to strong 11β-hydroxylating activity.)

On the other hand, our results show that 11-deoxycorticosterone—slightly but not significantly under these circumstances—displaces the specific binding of cortisol to rat adrenal mitochondria, suggesting that 11-deoxycorticosterone also binds to, and consequently might be metabolized by, CYP11B2, even if only to a small degree. This is in agreement with results obtained by other groups.[21,39]

If, as with certain receptors and channels (or other transport proteins), inhibitors were to classify, even to characterize, enzymes, the enzymes catalyzing the steps from 11-deoxycorticosterone to aldosterone would, according to our results, be either of a cortisol-sensitive (FS) or a cortisol-insensitive (FIS) type. Each type, or category, would comprise a minimum of one enzyme, although nothing can be said, according to these experiments, with regard to the number of enzymes within each category. Taking into account the enzymes already identified by molecular biology studies, CYP11B1 and CYP11B2 enzymes should be included in the FIS and FS categories, respectively.

Our mitochondrial preparation efficiently converts 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, 18-hydroxy-11-deoxycorticosterone, and 11,18-epoxycorticosterone to aldosterone. Paradoxically, the highest conversions were reached with the first two substrates, which is in appearance contradictory to the fact that both are above 18-hydroxycorticosterone on the biosynthetic pathway to aldosterone. This apparent discrepancy has been well known for many years[40] and has been assumed to be due to the higher polarity of the 18-hydroxylated steroid[18] or to its conformation.[40,31]

A complementary explanation would take into account the fact that only one enzyme catalizes the conversion of corticosterone to aldosterone. Under this assumption, once the intermediate 18-hydroxycorticosterone is formed, it might be converted directly to aldosterone without leaving the enzyme. Thus, when exogenously added, it would be unable to reach the right conformation to bind to the active site of the enzyme. This conformation could be 11,18-epoxycorticosterone–like. This last hypothesis is strongly supported by molecular biology studies which have shown that only one enzyme (18-hydroxylase/aldosterone synthase, CYP11B2) is involved in the transformation of corticosterone into aldosterone.[4–12]

Moreover, the step 11,18-epoxycorticosterone to aldosterone has not been assayed with cloned or isolated cytochromes P450.

It is well accepted that the main pathway from 11-deoxycorticosterone to aldosterone includes the intermediate sequence corticosterone and 18-hydroxycorticosterone.[1] However, alternative pathways have been proposed.[41,42] One of these alternatives includes the participation of 11,18-epoxycorticosterone.[31] Our results show that this steroid is transformed to aldosterone by rat adrenal zona glomerulosa mitochondria, and that this transformation is decreased by cortisol. On the other hand, 11,18-epoxycorticosterone showed a similar pattern for its effects on the metabolism of 11-deoxycorticosterone and corticosterone as cortisol (Tables 2 and 3). In effect, the addition of 11,18-epoxycorticosterone to incubation of 11-deoxycorticosterone with rat adrenal mitochondria slightly increased the production of corticosterone and decreased the production of aldosterone from the same substrate.

Similar to the main findings, our results show, therefore, that the conversion of 11,18-epoxycorticosterone to aldosterone is carried out by an FS enzyme (i.e. an enzyme belonging to the same category as CYP11B2).

Our results also raise another question: Is cortisol or one of its metabolites responsible for the effects here described?

The answer seems simple and points to cortisol. In effect, cortisol binds to adrenal mitochondria, and its binding is inhibited by sodium dithionite (data not shown), which suggests that the steroid is binding to the oxidized active form of cytochrome P450. In addition, the main metabolite isolated from incubations of tritiated cortisol with mitochondria seems to be 18-hydroxycortisol, but its yield is very poor (~4%) and its binding to adrenal mitochondria is lower than that of cortisol.[27] Finally, Lineweaver-Burk plots indicate that in rat adrenal mitochondria, just as in their bovine and duck counterparts,[27,28] cortisol behaves as a competitive inhibitor for the metabolism of corticosterone into aldosterone.

To the best of our knowledge, these results confirm for the first time through metabolic experiments, carried out on intact mitochondria, similar results obtained by molecular biology studies.[4–12]

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