ADRENAL 11-HYDROXYLASE ACTIVITY IN A HYPERCORTISOLEMIC NEW WORLD PRIMATE: ADAPTIVE INTRA-ADRENAL CHANGES

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Received July 7, 1987
Revised January 12, 1988

ABSTRACT

The squirrel monkey, a representative New World primate, has high plasma cortisol and aldosterone concentrations when compared to Old World primates. We measured adrenal mitochondrial 11-hydroxylase (11-OHase) activity in squirrel monkeys and in two representative Old World species (cynomolgus and rhesus macaques) in an effort to explain these elevated plasma glucocorticoid and mineralocorticoid levels. The activity of 11-OHase was 5-fold higher in the squirrel monkey than in the Old World species tested. Calculated 11-OHase Vmax was different in the squirrel monkey and the cynomolgus. However, the Km values were similar in the New World primate when compared to cynomolgus. The ability of metyrapone to block 11-OHase was less in the former than in the latter. The data are consistent with the hypothesis that the squirrel monkey adrenal cortex possesses an increased number of 11-hydroxylase enzyme units compared to that of Old World primate species, and is therefore more efficient in producing cortisol. This difference in 11-OHase activity in the squirrel monkey, in addition to other previously reported adrenal steroidogenic enzyme alterations, may be adaptive in nature, favoring increased cortisol and aldosterone production in this and possibly other New World primate species.

INTRODUCTION

The squirrel monkey (Saimiri sciureus), like other New World primates, has markedly elevated plasma cortisol concentrations, a result of low affinity glucocorticoid receptors and target tissue insensitivity to cortisol (1,2). Circulating cortisol in these species is mostly in the free form due to a markedly decreased level of cortisol-binding globulin (CBG) (2 – 4). Hypercortisolemia in squirrel monkeys is achieved by both an increased production and a decreased metabolic clearance rate of cortisol (5). The squirrel monkey has a slightly larger adrenal gland to body weight ratio than that of Old World species. Histologically the adrenal cortex of the squirrel monkey has a relatively larger zona fasciculata, and with this an elevated 21-hydroxylase activity and a low 17,20-desmolase

activity when compared to Old World primates, including man (5). These differences ultimately result in increased production of C21-hydroxylated steroids, primarily cortisol and presumably aldosterone (4,5). In fact, plasma aldosterone concentration is elevated in squirrel monkeys, approximately twice as high as that of the cynomolgus macaque (4).

The purpose of this study was to investigate further the enzymatic machinery of the highly efficient adrenal glands in the squirrel monkey by examining the activity of 11-hydroxylase, a principal enzyme in the biosynthesis of both cortisol and aldosterone.

EXPERIMENTAL

Materials

[^{3}H]Deoxycortisol, 50-60 Ci/mmol, and [^{14}C]cortisol, 50-60 mCi/mmol, were purchased from New England Nuclear, Boston MA. Nonradioactive 11-deoxycortisol was purchased from Steraloids, Wilton, NH. Thin-layer chromatography plates, Silica Gel LK6DF, were obtained from Whatman Inc., Clifton, NJ. Chromatography solvents were Fisher Scientific analytical grade, Fairlawn, NJ. Liquid scintillation cocktail (Aquasol) was purchased from New England Nuclear, Boston, MA. Glucose-6-phosphate dehydrogenase, NADPH, $MgCl_2$, and $CaCl_2$ were obtained from Sigma Chemical Co., St. Louis, MO. Metyrapone (Metopirone, 2-methyl-1,2,-bis-(3-pyradyl)-1-propanone, SU4885) was supplied by CIBA Pharmaceutical Company, Summit, NJ. Tris base and sucrose were purchased from Schwartz-Mann, Orangeburg, NJ.

Adrenal 11-Hydroxylase Activity Measurements

Mitochondrial Preparation: Whole, fresh adrenal glands from squirrel and cynomolgus monkeys were homogenized in 0.25 M sucrose at 4°C in a motor-driven Teflon/glass homogenizer (20 strokes). This was followed by 30 strokes with a ground glass/ground glass Dounce homogenizer. Tissue homogenates were centrifuged at 750 xg to remove nuclei and large cellular particles. Mitochondria were purified in the pellets of an 8,700 xg and 16,000 xg centrifugation. These pellets were pooled, washed once with Tris-$MgCl_2$ buffer (TRIS 50 mM, 5 mM $MgCl_2$, pH 7.4), centrifuged again at 16,000 xg, resuspended in Tris-$MgCl_2$ buffer, and frozen at -70°C in 500-μL aliquots until assayed. Mitochondrial protein was determined by the method of Lowry et al (6).

Assay Mixture: Each reaction volume was 100 μL and contained 5 μL propylene glycol, 11-[^{3}H]deoxycortisol (0.2 μM), nonradioactive 11-deoxycortisol (1.8 μM), NADPH (600 μM), glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (1.5 U/mL), and $CaCl_2$ (10 mM) (7). The buffer employed was Tris-$MgCl_2$, pH 7.4.

TABLE 1
ADRENAL 11-HYDROXYLASE ACTIVITY IN CYNOMOLGUS AND SQUIRREL MONKEYS WITH VARYING TIMES OF INCUBATION AND AMOUNTS OF MITOCHONDRIAL PROTEIN

Variable	11-OHase Activity[a] Cynomolgus	Squirrel Monkey
Time of Incubation (min)	pMoles product/mg protein	
2	68.1 ± 4.6	398.6 ± 30.9
4	120.9 ± 10.1	750.6 ± 55.5
6	146.8 ± 11.8	1192.9 ± 98.7
8	191.2 ± 20.0	1418.9 ± 162.1
Mitochondrial Protein (g)	pMoles product/min incubation	
10	0.4 ± 0.1	2.4 ± 0.3
25	1.2 ± 0.1	5.9 ± 0.5
50	1.4 ± 0.2	11.1 ± 1.2
100	2.5 ± 0.3	21.1 ± 1.9

[a]Substrate concentration is 2.0 μM 11-deoxycortisol. Values are given as mean ± SEM for two separate assays determined at three or four time points, each time point measured in triplicate in pmol product (cortisol) per min or g protein. When time of incubation was altered 25 mg of mitochondrial protein was used. When the amount of mitochondrial protein was changed, the incubation times were 4, 6, and 8 min.

Enzymatic Assay: The assay reactions were started by the addition of variable amounts of adrenal mitochondrial protein (11-hydroxylase) and allowed to incubate in a Dubnoff metabolic shaker at 37°C for 0-8 min. The reaction was terminated by the addition of 400 μL methanol containing 50 μg each of nonradioactive 11-deoxycortisol and cortisol. This solution (50 μL) was chromatographed with approximately 5000 dpm of [^{14}C]cortisol for estimation of recovery. TLC plates were developed twice with chloroform:ethanol (94:6, v:v). In this system cortisol has an rf of 0.24 and 11-deoxycortisol of 0.57. The activity of 11-hydroxylase was determined at three to five time points, each time point measured in triplicate. The activity of intact mitochondria at zero time was determined with each assay as a blank. To test for further differences in 11-OHase between the Old World and New World species, metyrapone, a selective inhibitor of 11-hydroxylase (8-16), was added to cynomolgus and squirrel monkey 11-OHase assays in concentrations ranging from 0.002 to 200 μM. Substrate concentration, i.e., 11-deoxycortisol, was kept constant at 2.0 μM in these studies.

Control

A single, pooled rhesus monkey adrenal mitochondrial preparation was run with each enzyme assay to control for intraassay and interassay variability. The intraassay and interassay coefficients of variation from the 11-OHase assay were 6% and 13%, respectively. To ensure that the endogenous steroid content of the mitochondria would not significantly decrease the specific activity of the substrates and thus falsely lower the observed activities, the mitochondrial

preparations from the pooled rhesus, cynomolgus, and squirrel monkey were assayed for 11-deoxycortisol using previously described radioimmunoassays (7, 17). The maximum endogenous steroid concentrations did not exceed 0.5% of the substrate concentration.

Derivative Formation

The identity of the product of the 11-OHase assay (cortisol) in the three species tested was confirmed by derivative formation using the product formed after the longest incubation. Cortisol was acetylated and then reduced with sodium borohydride in methanol (18). Each derivative was purified by TLC using chloroform:ETOH (94:6, v:v). The $^{3}H/^{14}C$ dpm ratios of each derivative did not change by more than 7% from the original ratio.

Statistical Analysis

Adrenal mitochondrial 11-OHase activities in the Old World and New World monkeys were compared with Student's t test. Estimates of Vmax and Km were made using the Lineweaver-Burk plot (Fig. 1) and the direct method of Cornish-Bowden and Eisenthal (19).

Calculations

The 11-OHase activity is expressed as picomoles (pMoles) of product cortisol produced per minute per milligram mitochondrial protein corrected for recovery.

RESULTS

The amount of cortisol produced in the 11-hydroxylase assay varied linearly with time of incubation and with changing mitochondrial protein (Table 1). The adrenal 11-hydroxylase activity in the squirrel monkey was approximately 5 times higher than that of the cynomolgus or rhesus macaques (Table 2).

TABLE 2

ADRENAL 11-HYDROXYLASE ACTIVITY IN ADRENAL GLANDS OBTAINED FROM RHESUS, CYNOMOLGUS, AND SQUIRRELS MONKEYS

Species	Enzyme Activity pMoles Cortisol/min/mg mitochondrial protein
Rhesus	39.3 ± 3.8
Cynomolgus	27.5 ± 3.2
Squirrel monkey	197.8 ± 14.4[a]

[a]$p<0.001$ compared to rhesus and cynomolgus. Values are given as mean ± SEM of triplicate determinations at three to five time points.
Substrate concentrations were 2.0 μM.

An assessment of the activity of 11-OHase for both the cynomolgus and squirrel monkey was made by changing substrate concentrations over a 20-fold range (0.25 μM to 5.0 μM). Km and Vmax were estimated to be 1.1 μM and 90 pmol/min/mg protein, respectively, for the cynomolgus and 1.1 μM and 350 pmol/min/mg protein for the squirrel monkey (Table 3, Fig. 1). Metyrapone suppressed both cynomolgus and squirrel monkey 11-OHase activity, with full inhibition occurring at a metyrapone concentration of 20 μM (Table 4). The estimated ED_{50} (20) for metyrapone inhibition was 0.024 μM for the cynomolgus and 0.043 μM for the squirrel monkey.

TABLE 3
ADRENAL 11-HYDROXYLASE ACTIVITY IN CYNOMOLGUS AND SQUIRREL MONKEYS WITH CHANGING SUBSTRATE CONCENTRATION

11-Deoxycortisol concentration (μM)	Cynomolgus	Squirrel Monkey
	pMoles Cortisol/min/mg mitochondrial protein[a]	
0.25	19.8 ± 2.6	72.5 ± 8.2
0.50	34.9 ± 2.9	106.9 ± 17.7
1.00	39.1 ± 2.9	165.0 ± 29.8
2.00	51.7 ± 4.5	222.7 ± 41.2
5.00	101.9 ± 16.5	309.8 ± 39.9

[a]Values given are mean ± SEM of two separate determinations at three different time points.

DISCUSSION

The degree of comparative adrenal 11-hydroxylase activity increase in the squirrel monkey is of the same magnitude as the elevated cortisol production rate in this species (approximately 5-fold) (5). This increase appears as a key to the mechanism of hypercortisolemia in the squirrel monkey. It can be explained by the presence of more enzyme units or by more efficient enzyme molecules in this primate. Since the estimated Km's for this enzyme were similar in the squirrel and cynomolgus monkey, and the Vmax was lower in the former, we suggest that the squirrel monkey adrenal contains more of the 11-hydroxylase enzyme, but that

TABLE 4
ADRENAL 11-HYDROXYLASE ACTIVITY IN THE PRESENCE OF INCREASING AMOUNTS OF THE 11-HYDROXYLASE INHIBITOR METYRAPONE

Metyrapone (μM)	Cynomolgus	Squirrel Monkey
	pMoles Cortisol/min/mg mitochondrial protein [a]	
0	69.3 ± 8.5	187.3 ± 15.9
0.002	59.4 ± 5.0	177.9 ± 22.0
0.01	49.3 ± 4.0	163.1 ± 17.1
0.02	38.9 ± 3.8	125.5 ± 15.0
0.1	10.3 ± 2.0	55.5 ± 8.4
0.2	2.2 ± 0.2	33.3 ± 4.5
2.0	2.0 ± 0.1	2.0 ± 0.2
20.0	0	0
200.0	0	0

[a] 11-Deoxycortisol concentration is 2.0 μM in all cases; 25 μg mitochondrial protein was used for all assays. Values given are mean ± SEM of two separate assays measured at three time points; 0 denotes levels below assay detection.

both the squirrel and cynomolgus monkey 11-hydroxylase enzymes have similar binding affinities for 11-deoxycortisol.

In vitro and in vivo studies have shown that metyrapone is a selective 11-hydroxylase inhibitor that diminishes the secretion of the 11-hydroxysteroids, including cortisol, corticosterone, and aldosterone, while having little if any effect on the 11-deoxycorticosteroids (12-15). The in vitro concentration of metyrapone needed to inhibit 50% of the 11-OHase was 2-fold greater in squirrel monkey mitochondria when compared to cynomolgus. However, this is probably not a sufficient change to suggest an alteration in the 11-hydroxylase enzyme active site for this inhibitor or presumably the substrate 11-deoxycortisol. This further supports the hypothesis that the adrenal mitochondrial 11-hydroxylase (or factors that influence its competency) may have changed in only a minor way in the New World primates to provide needed C_{21} steroids. In the squirrel monkey it appears that the main adaptation was to have increased the amount of 11-OHase in the adrenal, thus making this species more efficient in producing cortisol.

We believe that the observed enzymatic changes in the glucocorticoid-

mineralocorticoid pathway in the squirrel monkey may be part of a series of adaptive changes in New World primates (1, 21). The glucocorticoid "resistance" of these species may stem from the low affinity of the glucocorticoid receptor for glucocorticoids (2,18,22). To compensate for this, plasma ACTH levels increased (4,18), and adaptive changes in the efficiency of the squirrel monkey adrenal cortex to produce glucocorticoids occurred. Among these changes is a small increase in adrenal size, an increase in the relative size of the zona fasciculata, and an increase in the activity of three essential enzymes in the cortisol pathway, i.e., 17-hydroxylase, 21-hydroxylase (5), and 11-hydroxylase. The increased

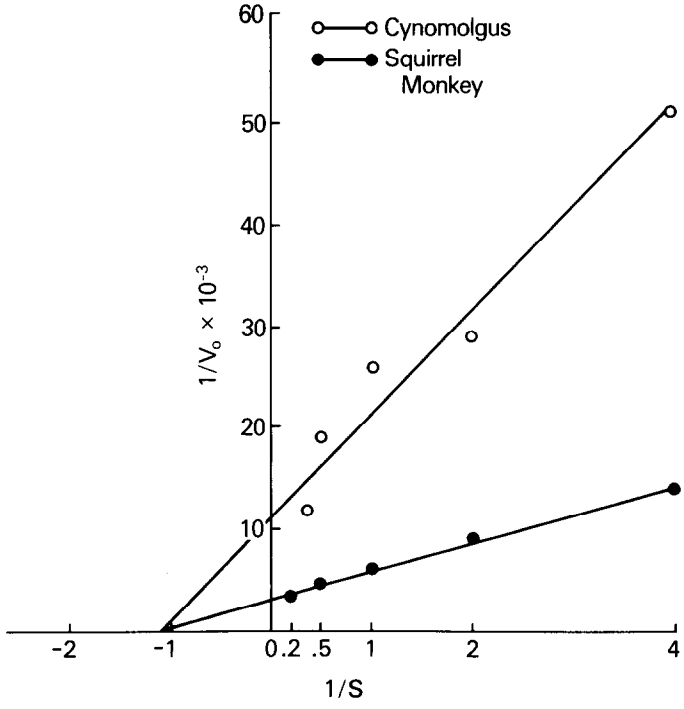

Figure 1: Lineweaver-Burk plot of data from experiments in which 11-deoxycortisol concentrations varied from 0.25 μM to 5.0 μM.

steroidosynthetic capacity of the tissue may have prevented the occurrence of excessive adrenal hypertrophy as would be expected from the high ACTH levels.

It is possible that the squirrel monkey and other New World species have adapted to the presence of both low affinity plasma cortisol-binding protein and low affinity tissue glucocorticoid receptors by: 1) increasing the relative size of the glucocorticoid-producing zone, 2) increasing the amount of 11-hydroxylase and other important enzymes to augment adrenal production of cortisol, and 3) by decreasing the substrate affinities of cortisol-catabolizing enzymes, causing a decrease in its metabolic clearance rate (21). Questions concerning the exact nature of the evolutionary adaptive changes in these glucocorticoid binding proteins and steroid-related enzymes will require further investigation

ACKNOWLEDGMENTS

The authors would like to acknowledge the effort and input of the late Dr. Mortimer B. Lipsett into initiation and development of this study; to thank Charles A. Brownley Jr. (CIBA Pharmaceutical Company) for generously supplying the metyrapone, and to Stefanie Emerson-May for her efficient assistance in typing the manuscript.

NOTE

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