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MITOCHONDRIAL STEROID METABOLISM IN THE INNER AND OUTER ZONES OF THE GUINEA-PIG ADRENAL CORTEX

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Summary—Previous investigations have demonstrated that cells isolated from the outer zone (zona fasciculata + zona glomerulosa) of the guinea-pig adrenal cortex produce far more cortisol than those from the inner zone (zona reticularis). Studies were carried out to compare mitochondrial steroid metabolism in the two zones. Protein and cytochrome P-450 concentrations were similar in outer and inner zone mitochondria. However, the rate of 11β-hydroxylation was significantly greater in the outer zone despite the fact that substrates for 11β-hydroxylation (11-deoxycortisol, 11-deoxycorticosterone) produced larger type I spectral changes in inner zone mitochondria. The apparent affinities of 11-deoxycortisol and 11-deoxycorticosterone for mitochondrial cytochrome(s) P-450 were similar in the two zones. In both inner and outer zone mitochondria, 11β-hydroxylation was inhibited by metyrapone but unaffected by aminoglutethimide. Cholesterol sidechain cleavage activity, measured as the rate of conversion of endogenous cholesterol to pregnenolone, was far greater in outer than inner zone mitochondria. Addition of exogenous cholesterol or 25-hydroxycholesterol to the mitochondrial preparations did not affect pregnenolone production in either zone. Addition of pregnenolone to outer zone mitochondria produced a reverse type I spectral change ($\Delta A_{420-390}$ nm), suggesting displacement of endogenous cholesterol from cytochrome P-450. In inner zone mitochondria, pregnenolone induced a difference spectrum ($\Delta A_{425-410}$ nm) similar to the reduced vs oxidized cytochrome b_5 spectrum. A b_5-like cytochrome was found to be present in the mitochondrial preparations. Prior reduction of the cytochrome with NADH eliminated the pregnenolone-induced spectral change in inner zone mitochondria but had no effect in outer zone preparations. The results suggest that differences in mitochondrial steroid metabolism between the inner and outer adrenocortical zones account in part for the differences in cortisol production by cells in each zone.

INTRODUCTION

The functions of the various anatomical zones of the adrenal cortex have been under investigation for several decades [1–4]. It has been clearly established that the outermost zone, the zona glomerulosa, is the site of aldosterone production, and therefore is primarily involved in mineralocorticoid function. However, there remains considerable uncertainty about the steroidogenic roles of the middle and innermost zones of the adrenal cortex, the zona fasciculata and zona reticularis, respectively. In particular, the relative importance of each zone with respect to glucocorticoid and androgen secretion needs to be resolved.

Recent observations [5–8] suggest that the zona fasciculata is the major site of cortisol production, at least in the guinea-pig. Cells isolated from the zona reticularis (inner zone) produced little or no cortisol whereas substantial amounts of cortisol were produced by cells from a combined zona fasciculata–zona glomerulosa (outer zone) preparation. We [9] and Obara and Strott[10] demonstrated higher rates of cholesterol sidechain cleavage in outer than inner zone mitochondria, suggesting that differences in mitochondrial steroid metabolism might be responsible, at least in part, for the zonal differences in cortisol production. The studies presented in this communication were done to further characterize steroid metabolism in mitochondrial preparations from the inner and outer zones of the guinea pig adrenal cortex.

EXPERIMENTAL

Adult male English Short Hair guinea-pigs, weighing approx. 700–800 g, were obtained from Camm Research Institute (Wayne, NJ) and used in all experiments. Animals were maintained under standardized conditions of light (0600–1800 h) and temperature (22°C) on a diet of Wayne Guinea Pig Diet and water *ad libitum*. Animals were killed by decapitation between 0800–0900 h. Adrenals were quickly removed and placed in cold 1.15% KCl containing 0.05 M Tris–HCl (pH 7.4). Adrenals were bisected longitudinally and the dark-brown inner zone, consisting primarily of zona reticularis, was gently dissected from the tan outer zone, which was comprised of the zona glomerulosa and zona fasciculata [11]. On the basis of steroid Δ^4-hydrogenase activity, which appears to reside solely in the inner zone [11], contamination of the outer zone preparations by inner zone material was less than 5%. Tissue from each zone was homogenized in 0.25 M sucrose containing

0.05 M Tris–HCl (pH 7.4) and mitochondria were obtained by differential centrifugation as previously described [12].

Mitochondrial 11β-hydroxylase activity was assayed as the rate of conversion of 11-deoxycortisol to cortisol, as described previously [13]. Cortisol was measured fluorometrically [14]. Cholesterol sidechain cleavage activity was determined as the rate of pregnenolone production by isolated adrenal mitochondria, with endogenous cholesterol as the substrate [13]. Cholesterol metabolism was initiated by the addition of 10 mM sodium isocitrate, and 4,4-dimethyl-2α-cyano-20-spirox-5-en-3-one (50 μM) a 3β-hydroxysteroid dehydrogenase inhibitor, was included in each flask to prevent the conversion of pregnenolone to progesterone [15]. In some experiments, sidechain cleavage assays were done after addition of exogenous cholesterol (in acetone) or 25-hydroxycholesterol (in ethanol) to the mitochondrial preparations. Pregnenolone was extracted from the incubation flasks with methylene dichloride and measured with a highly specific RIA [16, 17]. The pregnenolone antibody was obtained from Radioassay Systems Laboratories, Carson, CA, (Cat. No. 1670). Cross-reaction with cholesterol was less than 0.01%. All values were corrected for apparent pregnenolone levels at 0 incubation time (unincubated samples). The conversion of pregnenolone to progesterone by adrenal mitochondria was determined as described Neville and Engel[18] with modifications previously described [19]. Mitochondria (1.1 mg/protein) were incubated with pregnenolone (150 nmol) in a total volume of 2.5 ml and the formation of the conjugated 4-ene-3-one system determined by direct monitoring of the reaction mixture at 248 nm using an Amino DW-2a recording spectrophotometer. Cytochrome P-450 was measured as the dithionite-reduced CO complex [20], and cytochrome b_5 as described by Klingenberg[21]. Substrate-induced difference spectra in adrenal mitochondrial suspensions were obtained with an Aminco DW-2a recording spectrophotometer at 25°C, as previously described [22]. Mitochondrial protein was determined by the method of Lowry and coworkers[23].

RESULTS

Following dissection of guinea-pig adrenal glands into the chromatically distinct inner (zona reticularis) and outer (zona fasciculata plus zona glomerulosa) zones, approximately equal amounts of tissue were obtained from each zone (Table 1). Mitochondrial protein content and cytochrome P-450 concentrations were also similar in the inner and outer zones. Despite the similarity of cytochrome P-450 concentrations in the two zones, the rate of 11β-hydroxylation, a cytochrome P-450-dependent reaction, was significantly greater in the outer zone (Table 1). However, the magnitude of the type I spectral change produced by substrates (11-deoxycortisol, 11-deoxycorticosterone) for 11β-hydroxylation, indicative of binding to cytochrome P-450, was greater in inner than outer zone mitochondria (Table 1). The affinities of 11-deoxycortisol and 11-deoxycorticosterone for mitochondrial cytochrome(s) P-450, as determined by their spectral dissociation constants (K_s), were similar in the two zones (data not shown).

The concentrations of metyrapone, an 11β-hydroxylase inhibitor, producing a 50% decline in enzyme activity in the inner and outer zones, did not differ (Table 2). The inhibitor also induced the same type of spectral change in mitochondria from the two zones. Lambert and coworkers[24] recently reported that aminoglutethimide, a cholesterol sidechain cleavage inhibitor, also inhibited 11β-hydroxylation in guinea-pig adrenal cells. However, we found that concentrations of aminoglutethimide as high as 1.0 mM had no effects on 11β-hydroxylase activity in inner or outer zone adrenal mitochondria.

As previously reported [9,10], cholesterol sidechain cleavage activity, measured as the rate of conversion of endogenous cholesterol to pregnenolone, was far

Table 1. Protein and cytochrome(s) P-450 content, and cholesterol sidechain cleavage activity in inner and outer zone mitochondria*

	Inner zone	Outer zone
Adrenal weight (mg/pair)	216 ± 33	235 ± 29
Mitochondrial protein (mg/g tissue)	25.7 ± 2.8	27.8 ± 3.1
Cytochrome P-450 (nmol/mg protein)	0.67 ± 0.08	0.71 ± 0.09
11β-Hydroxylation (nmol cortisol/min × mg prot)	1.0 ± 0.2	1.8 ± 0.3†
11-Deoxycortisol Type I Spectrum (ΔA385–420 nm)(10^{-3})	23.9 ± 2.7	14.8 ± 1.9†
Cholesterol sidechain Cleavage (nmol pregnenolone/min × mg prot)		
− exogenous substrate	0.03 ± 0.01	0.16 ± 0.03†
+ cholesterol (100 μM)	0.02 ± 0.01	0.15 ± 0.02†
+ 25-OH-cholesterol (100 μM)	0.02 ± 0.01	0.14 ± 0.02†

*Values expressed as means ± SE of 6–8 observations.
†$P < 0.05$ (vs corresponding inner zone value).

Table 2. Metyrapone- and aminoglutethimide-induced spectral changes and effects on enzyme activities in inner and outer zone adrenal mitochondria

	Metyrapone		Aminoglutethimide	
	Inner	Outer	Inner	Outer
Spectral change ($A_{max-min}$)	425–395	425–395	430–410	430–395
11β-Hydroxylation‡ (I_{50})*	$7.8 \pm 1.3 \times 10^{-4}$ M	$8.7 \pm 1.1 \times 10^{-4}$ M	NE†	NE†
Cholesterol sidechain§ Cleavage (I_{50})*	NE†	NE†	$4.8 \pm 0.8 \times 10^{-5}$ M	$6.2 \pm 1.1 \times 10^{-5}$ M

*Concentration of inhibitor producing a 50% decrease in enzyme activity.
†No effect.
‡Measured as the rate of cortisol production (nmol/min × mg prot) from exogenous 11-deoxycortisol.
§Measured as the rate of pregnenolone formation (nmol/min × mg prot) from endogenous cholesterol.

greater in outer than inner zone mitochondria (Table 1). Addition of exogenous substrates (cholesterol, 25-hydroxycholesterol) to the mitochondrial preparations at concentrations as high as 100 μM, had no effects on pregnenolone production in either zone. Neither cholesterol nor 25-hydroxycholesterol, when added to the mitochondrial preparations, produced any demonstrable type of spectral change. Approximately the same concentrations of aminoglutethimide were required to produce a 50% decrease in cholesterol sidechain cleavage activity in inner and outer zone mitochondria (Table 2). The aminoglutethimide-induced difference spectrum was somewhat different in mitochondria from the two zones (Table 2). Metyrapone had no effect on sidechain cleavage activity in either zone.

The pregnenolone-induced reverse type I spectral change has been widely used as an index of the amount of cholesterol bound to cytochrome P-450 in adrenal mitochondria [25–27]. In outer zone adrenal mitochondria, pregnenolone produced the characteristic reverse type I spectrum, with a peak at about 420 nm and a trough at about 385–390 nm (Fig. 1), suggesting displacement of cholesterol from cytochrome P-450. However, in inner zone mitochondria, pregnenolone produced a different type of spectral change, one with a peak at about 425 nm and a trough at about 410 nm (Fig. 1). The latter resembles the reduced–oxidized difference spectrum of cytochrome b_5. In fact, addition of NADH to the mitochondrial preparations produced the same type of spectral change, indicating the presence of cytochrome b_5 or a b_5-like moiety. Its concentration was greater in inner than outer zone mitochondria (Fig. 1). After reduction of the b_5-like cytochrome with NADH, the pregnenolone-induced difference spectrum was no longer demonstrable in inner zone mitochondria but was unaffected in outer zone mitochondria (Fig. 2). NADH had no effect on the

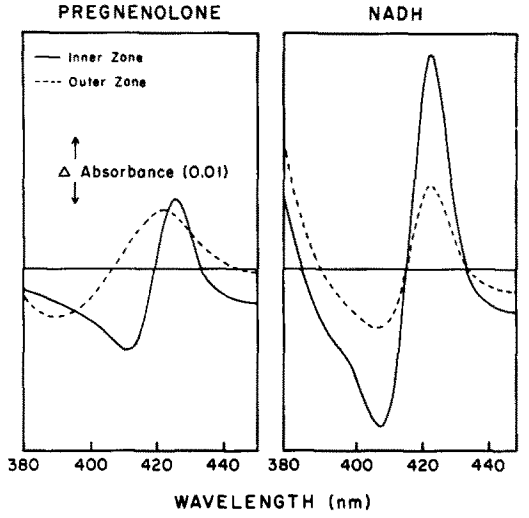

Fig. 1. Pregnenolone—and NADH—induced spectral changes in inner and outer zone adrenocortical mitochondria. Mitochondrial suspensions (1.1 mg prot/ml) were equally divided between two cuvettes and pregnenolone (150 nmol in 10 μl ethanol) or NADH (100 nmol in 10 μl sucrose–Tris buffer) was added to the sample cuvette. Vehicle alone was added to the reference cuvette. Spectra were recorded at 25°C and were corrected for the baselines of equal light absorbance.

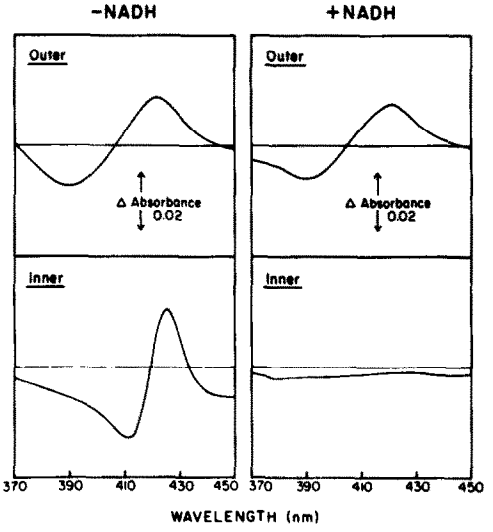

Fig. 2. Effects of NADH on the pregnenolone-induced difference spectra in inner and outer zone adrenocortical mitochondria. Mitochondrial suspensions were equally divided between two cuvettes at 25°C. NADH (100 nmol in 10 μl sucrose–Tris buffer) or buffer alone was added to both cuvettes and pregnenolone (150 nmol in 10 μl ethanol) was then added to the sample cuvette only. The reference cuvette received 10 μl of vehicle (ethanol). Spectra were recorded and corrected for the baselines of equal light absorbance.

Table 3. Effects of washing mitochondrial preparations from the inner and outer adrenocortical zones on 11β- and 21-hydroxylase activities and on the concentration of the b_5-like cytochrome

	11β-Hydroxylase‡	21-Hydroxylase§	b_5-like cytochrome
	(nmol/min × mg prot)		(nmol/mg prot)
Outer zone mitochondria			
Unwashed	1.7 ± 0.2	0.1 ± 0.1	0.19 ± 0.02
Washed × 1	1.9 ± 0.2	ND†	0.09 ± 0.02
Washed × 2	1.8 ± 0.2	ND†	0.09 ± 0.01
Washed × 3	1.9 ± 0.2	ND†	0.08 ± 0.02
Inner zone mitochondria			
Unwashed	0.9 ± 0.1	0.4 ± 0.1	0.32 ± 0.04
Washed × 1	1.1 ± 0.2	ND†	0.13 ± 0.02
Washed × 2	1.1 ± 0.2	ND†	0.12 ± 0.02
Washed × 3	1.0 ± 0.2	ND†	0.13 ± 0.02

*Values expressed as means ± SE of 4–6 observations.
†Not detectable.
‡Measured as the rate of conversion of 11-deoxycortisol to cortisol.
§Measured as the rate of conversion of 17α-hydroxyprogesterone to 11-deoxycortisol.

11-deoxycortisol-induced difference spectrum in mitochondria from either zone. The pregnenolone-induced spectral change in inner zone mitochondria was also eliminated by prior treatment of the mitochondria with 4,4-dimethyl-2α-cyano-20-spirox-5-en-3-one (L-625), a 3β-hydroxysteroid dehydrogenase inhibitor. L-625 had no effect on the reverse type I spectral change produced by pregnenolone in outer zone mitochondria.

To determine whether the presence of the b_5-like cytochrome in the mitochondrial preparations was the result of microsomal contamination, the effects of multiple washings of the mitochondrial preparations on cytochrome content and on the presence of 21-hydroxylase, a microsomal enzyme, were evaluated (Table 3). We routinely wash the mitochondria once prior to use in experiments. Unwashed mitochondrial preparations had measurable 21-hydroxylase activity, suggesting contamination by microsomes; activity was greater in inner than outer zone mitochondria. After the first wash, 21-hydroxylation was no longer demonstrable and the concentration of the b_5-like cytochrome declined by about 50% in mitochondria from both zones. Further washing of the mitochondria had no additional effects on cytochrome content. 11β-Hydroxylase activities were not significantly affected by any of the washes in either zone.

DISCUSSION

Recent observations [5–8] in several laboratories indicate that the inner zone (zona reticularis) of the guinea pig adrenal cortex produces very little cortisol and that substantially greater amounts are secreted by the outer zone (zona fasciculata plus zona glomerulosa). Differences in mitochondrial steroid metabolism appear to contribute to the differences in cortisol production by the two zones [9, 10]. Both 11β-hydroxylation and cholesterol sidechain cleavage, the rate-limiting step in steroidogenesis, proceed more rapidly in outer than inner zone mitochondria. The differences in sidechain cleavage activity may, in part, be attributable to a greater amount of cholesterol in outer zone mitochondria [9, 10], but addition of exogenous cholesterol or 25-hydroxycholesterol to the mitochondria had no effect on enzyme activity in either zone.

Mitochondrial concentrations of total cytochrome P-450, the terminal oxidase for 11β-hydroxylation and cholesterol sidechain cleavage, were similar in the inner and outer zones. In addition, the magnitude of the type I spectral change produced by substrates for 11β-hydroxylation, indicative of binding to cytochrome P-450, was greater in inner zone mitochondria. The affinities of the substrates for cytochrome P-450, as determined by their spectral dissociation constants (K_s), were similar in the two zones. Thus, there does not appear to be any correlation between substrate interactions with cytochrome P-450 and 11β-hydroxylase activities in inner and outer zone mitochondria. Further studies are needed to determine the mechanism(s) responsible for the higher 11β-hydroxylase activity in outer than inner zone mitochondria.

The effects of the monooxygenase inhibitors, metyrapone and aminoglutethimide, did not differ significantly in inner and outer zone mitochondria. Similar concentrations of metyrapone and aminoglutethimide were required for 50% inhibition of 11β-hydroxylase and cholesterol sidechain cleavage activities, respectively, in the two zones. Metyrapone had no effect on cholesterol sidechain cleavage in either zone, and in contrast to the observations of Lambert and coworkers[24], we found that aminoglutethimide had no effect on 11β-hydroxylation in outer or inner zone mitochondria. Thus, the enzyme inhibitors do not indicate any major differences between inner and outer zone mitochondrial monooxygenases.

The reverse type I spectral change produced by pregnenolone in guinea-pig outer zone mitochondria is similar to that previously found in adrenal mitochondria from other species [25–27]. The spectrum is

apparently caused by the displacement of cholesterol from cytochrome P-450 and has, therefore, been used as an indirect estimate of the amount of cytochrome P-450-bound cholesterol [25–27]. The pregnenolone-induced difference spectrum in inner zone mitochondria, however, differs considerably from that seen in other mitochondrial preparations and, in fact, closely resembles the spectral change produced in guinea-pig adrenal microsomal preparations [19]. In the latter preparations, pregnenolone serves as a substrate for the NAD^+-dependent, 3β-hydroxysteroid dehydrogenase, resulting in the formation of progesterone and NADH [19]. The NADH which is generated apparently reduces cytochrome b_5, since the resulting spectral change has a peak at about 425 nm and a trough at about 410 nm, identical to the reduced vs oxidized cytochrome b_5 spectrum [21]. A similar sequence of events may account for the pregnenolone-induced spectral change in inner zone adrenal mitochondria. It is known that there is 3β-hydroxysteroid dehydrogenase/isomerase activity in adrenal mitochondria as well as in microsomes [28–30], and we have found that guinea-pig adrenal mitochondria readily convert pregnenolone to progesterone (unpublished observations). Thus, the NADH produced during the metabolism of pregnenolone by inner zone mitochondria may be responsible for reduction of the b_5-like cytochrome, resulting in the observed spectral change. This hypothesis is supported by the observation that prior reduction of the cytochrome with NADH eliminated the pregnenolone-induced spectrum in inner zone mitochondria. In addition, inhibition of 3β-hydroxysteroid dehydrogenase activity with L-625 prevented the appearance of the spectral change, implicating pregnenolone metabolism in the development of the spectrum.

The reason(s) for the different types of spectral changes produced by pregnenolone in inner and outer zone mitochondria is probably related to the relative concentrations of cytochrome P-450-bound cholesterol and of the b_5-like cytochrome in each zone. Cholesterol concentrations as well as the rates of pregnenolone production from endogenous cholesterol are quite high in outer zone mitochondria [9, 10], suggesting that a substantial amount of cholesterol is associated with cytochrome P-450. Thus, the reverse type I spectral change caused by pregnenolone displacement of cholesterol from cytochrome P-450 in the outer zone is not surprising [25–27]. In inner zone mitochondria, by contrast, cholesterol concentrations are far lower and greater amounts of the b_5-like cytochrome are present. The very low rate of cholesterol sidechain cleavage in inner zone mitochondria suggests that there is very little cholesterol bound to cytochrome P-450 in that zone. Thus, the dominant spectral perturbation produced by pregnenolone in the inner zone probably results from the reduction of the b_5-like cytochrome.

Further studies are needed to more fully characterize the b_5-like cytochrome identified in the mitochondrial preparations. Its identity must be unequivocally established and its subcellular localization rigorously examined. A hemeprotein with the spectroscopic characteristics of cytochrome b_5 has previously been demonstrated in the outer mitochondrial membrane of rat liver [31–33] but not in adrenal mitochondrial preparations. Since our mitochondrial preparations were devoid of 21-hydroxylase activity, microsomal contamination does not appear to account for the presence of the b_5-like cytochrome. However, further investigation is needed before any definitive conclusions can be reached. The presence of an NADH-reducible cytochrome in adrenal mitochondria could play a role in steroidogenesis by providing a mechanism for the generation of NAD^+, an essential cofactor for 3β-hydroxysteroid dehydrogenase activity [18, 19]. Thus, additional studies on the localization and function of the b_5-like cytochrome seem warranted.

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