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19-HYDROXYLATION OF 18-HYDROXY-11-DEOXYCORTICOSTERONE BY ADRENAL MITOCHONDRIA PREPARED FROM VARIOUS ANIMAL SPECIES

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Summary—We have recently reported that bovine adrenocortical cytochrome P-450$_{11\beta}$ catalyzes 19-hydroxylation of 18-hydroxy-11-deoxycorticosterone (18(OH)DOC) in addition to 11β-hydroxylation of the steroid. In this report, we examine the presence of these two activities in 18(OH)DOC and 11β- and 18-hydroxylation activities on deoxycorticosterone (DOC) among the adrenal mitochondria prepared from man, ox, pig, rabbit, guinea-pig and rat. The results indicate that these animals could be classified into three groups with respect of these hydroxylation activities. Mitochondria of the first group comprising ox and pig showed rather high 19- and 11β-hydroxylation activities on 18(OH)DOC compared to the hydroxylation activities on DOC. Mitochondria prepared from the second group which comprised rabbit, guinea-pig and man showed low 19-hydroxylation activity on 18(OH)DOC, whereas the 11β-hydroxylation of 18(OH)DOC well occurred in these species. The last group comprising rat had very low activity both of 11β- and 19-hydroxylations when 18(OH)DOC was used as the substrate, whereas both 11β- and 18-hydroxylations of DOC were high in rat adrenal mitochondria.

No significant difference of these activities could be found between zona glomerulosa cells and zonae fasciculata-reticularis cells of bovine adrenal cortex, and between adrenal mitochondria from spontaneously hypertensive rat and those from WKY normotensive rat.

INTRODUCTION

It is well known that 18-hydroxy-11-deoxycorticosterone (18(OH)DOC) is produced in rat adrenal cortex and secreted from the gland [1, 2]. Implication of the steroid in human hypertension and rat experimental hypertension has been suggested by several investigators [3–5]. During a series of the studies on the metabolism of this steroid, we have found that when 18(OH)DOC was incubated with a reconstituted system of bovine adrenocortical cytochrome P-450$_{11\beta}$, a novel steroid was generated in addition to 11β-hydroxylated product, 18-hydroxycorticosterone (18(OH)B). From its mass spectra and [^{1}H]NMR spectrum, chemical structure of the novel steroid was suggested to be 18,19-dihydroxy-11-deoxycorticosterone (18,19(OH)$_2$DOC) [6, 7]. And this suggestion was further confirmed by comparing these spectra with those of chemically synthesized 18,19(OH)$_2$DOC [8]. To test physiological activity of this steroid, affinity of the steroid to aldosterone receptor and its mineralocorticoid activity were examined [9]. 18,19(OH)$_2$DOC did not show appreciable binding to the receptor and its relative potency as a mineralocorticoid was less than 1/8,000 that of deoxycorticosterone (DOC). In contrast, 18-hydroxy-19-nor-deoxycorticosterone, a putative metabolite of 18,19(OH)$_2$DOC, bound to the receptor with an affinity similar to that of 18(OH)DOC and its sodium retaining activity was also similar to that of 18(OH)DOC. Therefore we concluded that 18,19(OH)$_2$DOC could be a metabolic precursor of one of the mineralocorticoids.

In order to know whether 18,19(OH)$_2$DOC is generally produced in adrenal mitochondria, we measured in this report the generation of 18,19(OH)$_2$DOC from 18(OH)DOC using adrenal mitochondria prepared from various animal species. The reactions were conducted in the incubation system composed of mitochondria, bovine adrenodoxin and bovine adrenodoxin reductase.

EXPERIMENTAL

Reagent

Chemical synthesis of 18,19(OH)$_2$DOC was described in the previous paper [8]. 16α,18-Dihydroxy-11-deoxycorticosterone was a generous gift from Dr D. N. Kirk of Westfield College, London. Most other steroids were purchased from Makor Chemicals (Jerusalem, Israel) and from Sigma (U.S.A.). NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase (yeast) were obtained from Oriental Yeast Co. Ltd (Japan). All other chemicals of the highest purity available were obtained from commercial sources. Bovine adrenodoxin and adrenodoxin reductase were purified as described elsewhere [10, 11].

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Animals

Sprague–Dawley rats and male Hartley guinea pigs were purchased from Kiwa Animals, Japan. Male spontaneously hypertensive rats (SHR) and WKY control rats were obtained from Charles River, Japan. They were maintained by standard rat chow. Water was given *ad libitum*. The measurement of blood pressure of the rat was carried out sphygmomanometrically. Male Japanese white rabbits were obtained from Keari, Japan.

Preparation of adrenal mitochondria

Bovine and porcine adrenal glands were obtained from a local slaughterhouse. Human adrenal glands were obtained at autopsy within 24 h of death. No pathological change was detected in these adrenal glands. The adrenal gland was bisected, the medullary tissue was removed and the cortical tissue was obtained. The cortex was homogenized in 4 vol of 0.25 M sucrose and the homogenate was centrifuged at 800 *g* for 10 min. The supernatant was further centrifuged at 9,000 *g* for 20 min. The mitochondrial precipitate thus obtained was washed once with 0.25 M sucrose. In the case of rat, guinea pig and rabbit, the adrenal glands were obtained within 1 h of death of the animal and were homogenized in 0.25 M sucrose. The homogenate was centrifuged as described above. The mitochondrial pellets were stored at −80°C until use.

Determination of cytochrome P-450 content

The concentration of cytochrome P-450 was estimated from absorbance difference at 450 nm between CO-reduced and reduced form, using millimolar absorption coefficient of 91 cm^{-1} mM^{-1} [12].

Zonal dissection of bovine adrenal cortex

Methods of separation of the bovine adrenal cortex into zona glomerulosa and zonae fasciculata-reticularis were previously described by Yagi *et al.*[13].

Hydroxylation of steroid

Steroid hydroxylase activity was measured as described previously [7, 8]. Mitochondrial pellets suspended in 0.25 M sucrose were mixed with one ninth volume of 10% sodium cholate before use. An aliquot (50 μl) of this suspension was added to 440 μl of reaction mixture containing steroid (50 nmol), glucose-6-phosphate (10 μmol), glucose-6-phosphate dehydrogenase (0.5 unit), $MgCl_2$ (3 mM), adrenodoxin (15 nmol), adrenodoxin reductase (1 nmol) and Tris–HCl, pH 7.4 (50 mM). After pre-incubation for 45 s at 37°C, the reaction was initiated by addition of 10 μl of 5 mM NADPH, allowed to continue for 4 min, and terminated by addition of 0.5 ml of ethanol. The steroids were extracted with 5 ml of dichloromethane, and the solvent was evaporated under a stream of nitrogen. To estimate the production of 18(OH)B and 18,19$(OH)_2$DOC from 18(OH)DOC, the dried extract dissolved in 35% (v/v) dioxane in distilled water was injected into a reverse-phase column (Chemcosorb ODS.H.7 μ, 4 × 300 mm, Chemco Co. Ltd, Japan) and the steroids were eluted with the same solvent. To estimate the formation of corticosterone and 18(OH)DOC from DOC, the dried extract dissolved in 60% (v/v) methanol in water was injected into the same column and the steroids were eluted with 60% methanol. Steroids were estimated by monitoring the absorbance at 254 nm of the eluent. All assays were conducted in duplicate. Unless otherwise stated, the results were expressed as the means of the duplicate assays. A high-performance liquid chromatography system employed was made up by a LDC liquid chromatograph system (Florida, U.S.A.) equipped with a Constametric III pump, a UV monitor Model 1203 and a syringe loading sample injector Model 7120 (Rheodyne, Berkeley, U.S.A.).

Protein determination

Protein was determined by the microbiuret method of Bensadoun and Weinstein[14] with crystalline bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Steroid hydroxylation activity of human adrenocortical mitochondria

As shown in Table 1, human adrenocortical mitochondria contained 0.17–0.25 nmol of cytochrome P-450/mg protein and this specific content of the cytochrome P-450 seems to be lower than those of the other animals examined (Table 2). However, the human mitochondria exhibited a considerably high level of 11β-hydroxylation activity on DOC (8.0–13.2 nmol corticosterone formed/min/mg protein). From these results it may be assumed that human adrenocortical mitochondria possess a rather large amount of cytochrome P-$450_{11\beta}$, or the specific activity of human adrenal cytochrome P-$450_{11\beta}$ is rather high.

In the incubation of 18(OH)DOC with the human adrenocortical mitochondria, a trace amount of 18,19$(OH)_2$DOC and a rather large amount of 18(OH)B (0.6–1.4 nmol/min/mg protein) were produced. These results indicate that human adrenal cytochrome P-$450_{11\beta}$ catalyzes rather efficient 11β-hydroxylation, while the activity of 19-hydroxylation is very low.

Dale and Melby [15] reported that 18(OH)DOC was converted not only to 18(OH)B but also to 16α,18-dihydroxy-11-deoxycorticosterone during the incubation with human adrenal tissue. However, we could not detect 16α,18-dihydroxy-11-deoxycorticosterone in the incubation. The absence of the 16α-hydroxylated product may be due to the absence of 16α-hydroxylase in the mitochondrial fraction.

Table 1. Hydroxylation of DOC or 18(OH)DOC by human adrenocortical mitochondria*

Number of experiment (P-450 content)	Activity (nmol product formed/min/mg protein)			
	DOC→B	DOC→ 18(OH)DOC	18(OH)DOC→ 18(OH)B	18(OH)DOC→ 18,19(OH)$_2$DOC
1 (0.17)†	8.0‡	0.6	1.3	Not detected
	(100)	(7.5)	(16.3)	(—)
2 (0.25)	12.2	0.7	1.4	Not detected
	(100)	(5.7)	(11.5)	(—)
3 (0.13)	9.0	0.3	0.6	0.1
	(100)	(3.3)	(6.7)	(1.1)
4 (0.22)	13.2	0.6	0.6	0.1
	(100)	(4.5)	(4.5)	(1.0)

*In each experiment, the mitochondria were prepared from one human adrenal gland obtained within 24 h of death. The results were expressed as the means of the duplicate assays.
†nmol P-450/mg protein.
‡The numbers in parentheses are percentages of 11β-hydroxylase activity on DOC.

Variation of steroid hydroxylation activity among various animal species

To know whether the 19-hydroxylation of 18(OH)DOC generally takes place in the adrenal mitochondria, we measured the hydroxylase activity of adrenal mitochondria prepared from various animal species. As shown in Table 2, hydroxylation activity on DOC and 18(OH)DOC was much varied among animal species. In terms of the ratio of production of 18(OH)B or 18,19(OH)$_2$DOC from 18(OH)DOC to that of corticosterone from DOC, these animals appear to be classified into three groups. The first group comprises pig and ox. When 18(OH)DOC was incubated with adrenocortical mitochondria of these animals, a rather high hydroxylase activity both at 11β- and 19-positions was found; in the case of porcine adrenals, the 11β-hydroxylation of 18(OH)DOC was 43.1% of the rate of the corticosterone formation from DOC and the 19-hydroxylation of 18(OH)DOC was 20.8%. These hydroxylation activities of bovine adrenocortical mitochondria were 15.7% at 11β-position and 15.1% at 19-position, respectively.

The second group comprises guinea-pig, rabbit and man. Adrenal mitochondria of these animals catalyzed hydroxylation exclusively at 11β-position when 18(OH)DOC was used as the substrate. Thus the rate of formation of 18,19(OH)$_2$DOC from 18(OH)DOC was very low compared to that of 18(OH)B from 18(OH)DOC.

The last group comprises rat. The rat adrenal mitochondria scarcely catalyzed the hydroxylation of 18(OH)DOC both at 11β- and 19-position compared to their rather high hydroxylation activity on DOC at 18- and 11β-positions.

Steroid hydroxylation activity of adrenal mitochondria prepared from spontaneously hypertensive rats

Since all the hydroxylation activities we have measured in this study are closely related to metabolism of mineralocorticoids, it would be interesting to see whether these activities of spontaneously hypertensive rats (SHR) are different from those of wild type rats (WKY). Table 3 presents these activities of SHR rats and WKY rats. A tendency of rat adrenal mitochondria described above, a low hydroxylation activity on 18(OH)DOC and a rather high hydroxylation activity on DOC, was also observed in these

Table 2. Hydroxylation of DOC or 18(OH)DOC by adrenal mitochondria from various animal species

Animal species (P-450 content) (nmol P-450/mg protein)	Activity (nmol product formed/min/mg protein)			
	DOC→B	DOC→ 18(OH)DOC	18(OH)DOC→ 18(OH)B	18(OH)DOC→ 18,19(OH)$_2$DOC
Porcine[b]	7.2	0.4	3.1	1.5
(1.19)	(100)[a]	(5.6)	(43.1)	(20.8)
Bovine[b]	16.8 ± 1.35*	1.43 ± 0.74	2.67 ± 0.45	2.61 ± 0.76
(0.76 ± 0.065)*	(100)	(8.1)	(15.7)	(15.1)
Guinea-pig†	4.1	0.4	0.5	0.2
(0.66)	(100)	(9.8)	(12.2)	(4.9)
Rabbit‡	3.0	0.2	0.5	0.1
(0.37)	(100)	(6.7)	(16.7)	(3.3)
Rat§	30.3 ± 6.9‖	15.2 ± 3.1	0.32 ± 0.01	0.18 ± 0.06
(0.83 ± 0.12)‖	(100)	(51.8)	(1.1)	(0.6)

[a]The numbers in parentheses are percentages of 11β-hydroxylase activity on DOC.
[b]Mitochondria were prepared from whole adrenal cortex.
*The experiments were conducted four times, and the results were expressed as mean ± SD.
†The mitochondria were prepared from adrenal glands of 10 male Hartley guinea-pigs.
‡The mitochondria were prepared from adrenal glands of 5 male Japanese white rabbits.
§Rats of Sprague–Dawley strain, male and female, 20-weeks old, were used.
‖The experiments were conducted three times, and the results were expressed as means ± SD.

Table 3. Hydroxylation of DOC or 18(OH)DOC by adrenocortical mitochondria of spontaneously hypertensive rat

Strain (P-450 content) (nmol P-450/mg protein)	Activity (nmol product formed/min/mg protein)			
	DOC→B	DOC→ 18(OH)DOC	18(OH)DOC→ 18(OH)B	18(OH)DOC→ 18,19(OH)$_2$DOC
SHR*	53.4	33.2	0.4	0.1
(0.93)	(100)	(62.2)	(0.7)	(0.2)
WKY†	66.4	41.7	0.3	0.1
(0.91)	(100)	(62.8)	(0.5)	(0.2)

*15 Male SHR rats, 95-days old, were used. Their blood pressure was 161 ± 9 mmHg (mean ± SD), which was measured sphygmomanometrically.
†15 Male WKY rats, 95-days old, were used. Their blood pressure was 122 ± 7 mmHg (mean ± SD).

strains of rats. Moreover there seemed to be no significant difference in terms of these hydroxylation activities between SHR and WKY rats.

Zonal distribution of 19-hydroxylation activity on 18(OH)DOC in bovine adrenal cortex

Histochemically adrenal cortex is composed of three zones; zona glomerulosa, zona fasciculata and zona reticularis. It has been known that aldosterone is secreted from zona glomerulosa whereas glucocorticoids and sex hormones are secreted from zonae fasciculata-reticularis [13, 16–19]. Moreover, Müller [20] has reported the zonal specificity of 11β-hydroxylation of 18(OH)DOC in rat adrenal tissues. Therefore we compared the hydroxylase activity of the mitochondria from zona glomerulosa of bovine adrenal cortex with that from zonae fasciculata-reticularis. As shown in Table 4, the specific content of cytochrome P-450 in each zone seems to be lower than that of mitochondria prepared from whole bovine adrenal cortex as described in Table 2. This lower content may be explained by the unstable nature of cytochrome P-450, a part of which would be converted into cytochrome P-420 during the zonal dissection of the adrenal cortex. Provided that the rate of production of corticosterone from DOC was taken as 100%, the rates of production of 18(OH)B from 18(OH)DOC appeared to be similar between these two zones; 22.7 and 21.4% for zona glomerulosa and zonae fasciculata-reticularis, respectively. The rates of production of 18,19(OH)$_2$DOC from 18(OH)DOC were also similar between these zones (18.0 and 18.2% for zona glomerulosa and zonae fasciculata-reticularis, respectively). These results may indicate that no significant difference exists among adrenocortical three zones in terms of P-450$_{11\beta}$-catalyzed reaction. These observations agree with our previous report [13].

CONCLUSION

Taken together, it can be concluded that the hydroxylation activity on 18(OH)DOC catalyzed by adrenal mitochondria much varied among animal species. No significant differentiation was found in terms of the cytochrome P-450$_{11\beta}$-catalyzed reactions among three zones of adrenal cortex, as shown in the experiments using zona glomerulosa and zonae fasciculata-reticularis of bovine adrenal cortex.

In our HPLC profiles of the products obtained from the incubation of DOC or 18(OH)DOC with adrenal mitochondria of various animals, hitherto unidentified peaks other than those described in this paper have also been observed. Our present investigation is focused on chemical identification of these unidentified corticosteroids.

The metabolic pathway of 18(OH)DOC and the biological role of the metabolites *in vivo* remain to be explored.

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Table 4. Hydroxylation of DOC or 18(OH)DOC by bovine adrenocortical mitochondria*

Tissue† (P-450 content) (nmol P-450/mg protein)	Activity (nmol product formed/min/mg protein)			
	DOC→B	DOC→ 18(OH)DOC	18(OH)DOC→ 18(OH)B	18(OH)DOC→ 18,19(OH)$_2$DOC
Zona glomerulosa	15.0	1.1	3.4	2.7
(0.48)	(100)‡	(7.3)	(22.7)	(18.0)
Zonae fasciculata and reticularis (0.36)	15.4	1.0	3.3	2.8
	(100)	(6.5)	(21.4)	(18.2)

*The experiments were conducted two times and the errors were always within 10% of the mean.
†Zonal dissection of the adrenal cortex was carried out as described in Experimental.
‡The numbers in parentheses are percentages of 11β-hydroxylase activity on DOC.

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