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The Final Step of Aldosterone Biosynthesis is Catalyzed by an NADPH-dependent and Molecular Oxygen-requiring Enzyme

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Using sonicated mitochondria fraction prepared from bovine adrenal glomerulosa cells, aldosterone biosynthesis from 18-hydroxycorticosterone was examined as its final step, as production of [^{3}H]-aldosterone from [^{3}H]-corticosterone was strongly reduced by addition of non-radioactive 18-hydroxycorticosterone during the incubation. Significant conversion of 18-hydroxycorticosterone to aldosterone by the mitochondria sonicate was observed in the presence of NADPH, but not $NADP^+$. This reaction was almost completely inhibited in the atmosphere of 100 % carbon monoxide in the presence of either $NADP^+$, or NAD^+, and significantly reduced in the mixture of carbon monoxide and oxygen (90:10) in the presence of NADPH. Several drugs, such as SU compounds, spironolactone, amphenone B and SKF 525A which affect cytochrome P-450 blocked production of aldosterone from 18-hydroxycorticosterone. From these results, we conclude that a mixed function oxidase involving a cytochrome P-450 is engaged in the final course of aldosterone biosynthesis.

INTRODUCTION

Aldosterone, the most potent mineral corticoid, is secreted from adrenal glomerulosa cells. Although many studies on biochemistry of aldosterone have been performed, biosynthetic pathway of aldosterone from corticosterone is not fully elucidated. As found in

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some textbooks of endocrinology (1,2) and a related review (3), corticosterone is converted to 18-hydroxycorticosterone by 18-hydroxylase and then 18-hydroxycorticosterone is further converted to aldosterone by so-called "18-hydroxysteroid dehydrogenase". However, there have been few evidences that support the concept of dehydrogenation for the synthesis of aldosterone. On the other hand, there are several arguments about nature of the enzyme which catalyzes the final reaction of aldosterone biosynthesis and about the role of 18-hydroxycorticosterone as an obligatory intermediate (4). Ulick postulated mainly from clinical observations that the last steps for aldosterone biosynthesis are catalyzed by two types of mixed function oxidase (5,6), though no direct biochemical evidence was obtained.

In the present communication, we report that the final reaction for aldosterone biosynthesis is catalyzed by an enzyme which is NADPH-dependent and requires molecular oxygen.

MATERIALS AND METHODS

Steroids 18-Hydroxycorticosterone and aldosterone were obtained from Makor Chem. Ltd., Israel. [1,2-^{3}H]-Corticosterone (S.A. 60 Ci/mmol) and [1,2-^{3}H]-18-hydroxycorticosterone (S.A. 60 Ci/mmol) were purchased from Amersham, U. K. Purities of the radioactive steroids were confirmed by thin layer chromatography immediately before use.

Preparation of Adrenal Mitochondria Fresh bovine adrenal glands were obtained from a slaughterhouse, and their glomerulosa cells were separated and homogenized with a loose fitting Teflon-glass homogenizer in Tris-(10 mM)-HCl-buffered 0.33 M sucrose solution (pH 7.4). After centrifugation of the homogenates at 800 x g for 20 min, the supernatant fluid was centrifuged at 10,000 x g for 20 min to obtain mitochondrial fraction as the precipitate. The precipitate was then suspended in the same buffer and was centrifuged again at 10,000 x g for 20 min. The washed mitochondrial pellet was suspended in the buffer. The mitochondrial fraction was subjected to 20 Kcycle/sec sonication for 5 min at 0 to 5°C, and was used as mitochondrial sonicate.

Incubation Radioactive steroids were dissolved in ethanol and appropriate amount of the solution was transferred to an incubation flask. Two drops of propylene glycol per flask were added and then the ethanol was removed under reduced pressure shortly before incubation. Incubation mixture generally consisted of the radioactive steroid (0.3 μCi), the mitochondrial preparation and cofactor (final concentration 240 μM) in 0.33 M sucrose solution buffered with Tris-HCl at pH 7.4. The final volume was adjusted as 5 ml. The mixture was incubated at 37°C for 60 min in an aerobic atmosphere, unless otherwise mentioned.

Quantitation of Aldosterone Synthesis Immediately after the incubation, reaction was stopped by addition of 15 ml of methylenechloride and the mixture was vigorously shaken to extract the steroids. The methylenechloride layer was collected and, to the extract, non-radioactive 18-hydroxycorticosterone and aldosterone were added as carriers. The extract was dried with anhydrous sodium sulfate and was evaporated to dryness under reduced pressure. An aliquot of the steroid extract was chromatographed on a thin layer plate coated with silica gel GF_{254} (Merck A.G., Germany), using solvent system of benzene-acetone (1:1, v/v). After development of the thin layer plates, the spots of the carrier steroids on the chromatograms were detected under ultraviolet light at 254 nm. The detected spots were scraped off and packed into a small glass column. Each steroid was extracted by a mixture of chloroform-ethanol (1:1, v/v) from the absorbent. Radioactive product was identified as aldosterone by recrystallization. The radioactivity was measured with a liquid scintillation spectrometer. Aldosterone production was expressed as fmol/mg protein for 60 min, after radioactivity of aldosterone derived from [^{3}H]-18-hydroxycorticosterone or [^{3}H]-corticosterone was converted into fmol on the basis of its specific radioactivity.

Protein Assay Protein concentrations of the mitochondrial preparations were determined by a kit of the Bio-Rad protein assay, using bovine gamma globulin as the standard.

RESULTS AND DISCUSSION

18-Hydroxycorticosterone as an Intermediate for Aldosterone Synthesis from Corticosterone Production of radioactive aldosterone from [^{3}H]-corticosterone by the sonicated mitochondria was reduced by addition of non-radioactive 18-hydroxycorticosterone in a dose-dependent manner (Table 1). This result indicates that the tritiated 18-hydroxycorticosterone derived from [^{3}H]-corticosterone was diluted with the exogenously added 18-hydroxycorticosterone during the incubation,

TABLE 1
Effect of 18-hydroxycorticosterone on production of [^{3}H]-aldosterone from [^{3}H]-corticosterone

18-Hydroxycorticosterone	Aldosterone Production (fmol/mg protein/60 min)	
None	11.8	(100)*
1×10^{-10} M	7.4	(63)
1×10^{-7} M	2.9	(25)
1×10^{-4} M	1.3	(11)

* Relative ratio (%) to the yield without 18-hydroxycorticosterone

and apparent reduction of [^{3}H]-aldosterone formation was caused. Furthermore, radioactive aldosterone was synthesized by the sonicated mitochondria from [^{3}H]-18-hydroxycorticosterone in the presence of NADPH under an aerobic condition, as shown in Table 3. From these results, 18-hydroxycorticosterone was considered as an intermediate for aldosterone synthesis from corticosterone, and accordingly, conversion of 18-hydroxycorticosterone to aldosterone was regarded as the final step of aldosterone synthesis. On the other hand, it has been reported that 18-hydroxycorticosterone was doubted as the intermediate, because corticosterone was more efficiently transformed *in vitro* to aldosterone than 18-hydroxycorticosterone itself (7,8). This seems to be due to limited accessibility of the re-introduced 18-hydroxycorticosterone as substrate to catalytic site(s) of the enzyme system.

Cofactor Requirement for Aldosterone Synthesis from 18-Hydroxycorticosterone

a) Intact Mitochondria According to our time-course study of aldosterone production from 18-hydroxycorticosterone by the intact mitochondria, radioactive aldo-

TABLE 2
Influence of cofactors upon aldosterone production from 18-hydroxycorticosterone by intact and sonicated mitochondrial preparations

Mitochondria	Cofactor	Aldosterone Production (fmol/mg protein/60 min)	
Intact			
	malate	12.1	(100)*
	NADPH	2.1	(17)
	$NADP^+$	0.3	(2)
Sonicated			
	NADPH	11.6	(100)**
	$NADP^+$	1.9	(16)

* Relative ratio (%) to the yield obtained with $NADP^+$ and malate
** Relative ratio (%) to the yield obtained with NADPH in case of sonicated mitochondria

sterone was produced almost linearly up to 60 min. As shown in Table 2, aldosterone production was enhanced in the presence of malate, but neither NADPH nor $NADP^+$, that suggests dependency of aldosterone synthesis upon intramitochondrial generation of NADPH.

b) Sonicated Mitochondria To examine requirement of cofactor furthermore, the sonicated adrenal mitochondria were incubated in the presence of various cofactors. Increased production of aldosterone was observed by exogenous administration of NADPH but not $NADP^+$ (Table 2). This suggests that the final reaction for aldosterone synthesis is catalyzed by an NADPH-requiring enzyme system, in agreement with the report of Marusic et al. (9).

Effects of Incubation Atmospheres upon Aldosterone Synthesis from 18-Hydroxycorticosterone Formation of aldosterone from 18-hydroxycorticosterone by the sonicated mitochondria was almost completely blocked even in the presence of NAD^+ and $NADP^+$, when the

TABLE 3
Effects of gas phases and cofactors upon aldosterone production from 18-hydroxycorticosterone

Gas Phase (%)			Cofactor	Aldosterone Production (fmol/mg protein/60 min)	
O_2	CO	Ar			
10	0	90	NADPH	13.5	(100)*
10	90	0	NADPH	4.4	(33)
0	100	0	$NADP^+$	0.8	(6)
0	100	0	NAD^+	0.8	(6)

* Relative ratio (%) to the yield obtained in the presence of NADPH under the atmosphere of O_2 and Ar (10:90)

atmosphere was replaced with 100 % carbon monoxide, that are favorable for enzymic dehydrogenation. Furthermore, when incubated in the atmosphere of the mixture of CO and O_2 (90:10), aldosterone synthesis was partially but significantly inhibited, in comparison with the yield obtained in the atmosphere of Ar and O_2 (90:10), as shown in Table 3.

Effects of Cytochrome P-450 Inhibitors upon Aldosterone Synthesis from 18-Hydroxycorticosterone Effects of several compounds which affect cytochrome P-450 (10,11) were examined upon conversion of 18-hydroxycorticosterone to aldosterone by the sonicated mitochondria.

TABLE 4
Effects of P-450 inhibitors upon conversion of 18-hydroxycorticosterone to aldosterone

Inhibitor*	Aldosterone Production (fmol/mg protein/60 min)	Relative Rate (%)
None	16.8	100
SU 4885	2.9	17
SU 8000	3.6	21
SU 10603	1.6	10
SKF 525A	2.2	13
Amphenone B	3.6	21
Spironolactone	1.5	9

* Concentration of each inhibitor was 5 mM.

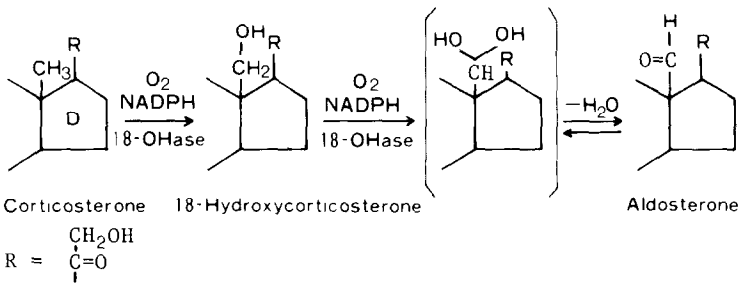

Fig. 1. Biosynthetic pathway of aldosterone from corticosterone and related enzymology

All these compounds markedly inhibited production of aldosterone from 18-hydroxycorticosterone in various degrees (Table 4), in agreement with the previous report (12). These compounds also inhibited the conversion of corticosterone to 18-hydroxycorticosterone and to aldosterone (data not shown). These results obtained in the present experiment suggest that the final reaction occured as a cytochrome P-450 related oxygenation but not dehydrogenation. Furthermore, conversion of corticosterone to aldosterone is suggested as composed of the two following steps; from corticosterone to 18-hydroxycorticosterone and finally from 18-hydroxycorticosterone to aldosterone. Therefore, we postulate the biosynthetic pathway of aldosterone and related enzymology, as shown in Fig. 1.

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