Journal of Steroid Biochemistry, 1978, Vol. 9, pp. 967–972. Pergamon Press. Printed in Great Britain

ANGIOTENSIN STIMULATES BOTH EARLY AND LATE STEPS IN ALDOSTERONE BIOSYNTHESIS IN ISOLATED BOVINE GLOMERULOSA CELLS*†

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(*Received* 28 *November* 1977)

SUMMARY

The present study was designed to examine the effect of angiotensin on both the early and the late phases of aldosterone biosynthesis. In order to isolate the early phase (the formation of pregnenolone), it was necessary to identify an agent that would inhibit the 3β-hydroxysteroid dehydrogenase, 4-5-ene-isomerase enzyme system which facilitates the conversion of pregnenolone to progesterone. Such an agent was found in trilostane. Treatment of zona glomerulosa cells with trilostane resulted in accumulation of pregnenolone, and the addition of angiotensin increased the accumulation of pregnenolone still further. This observation demonstrates that angiotensin stimulates aldosterone biosynthesis at a point prior to pregnenolone formation.

The late phase of aldosterone biosynthesis was isolated by using aminoglutethimide to inhibit the formation of pregnenolone by cells of the zona glomerulosa. Aldosterone was formed when aldosterone precursors occurring in the biosynthetic pathway from pregnenolone onward were added. When angiotensin was added along with deoxycorticosterone, the conversion of deoxycorticosterone to aldosterone was enhanced ($P < 0.05$). This observation demonstrates that angiotensin stimulates aldosterone formation at a point distal to deoxycorticosterone formation. Thus, angiotensin stimulates aldosterone biosynthesis at at least 2 independent points, one early and one late in the biosynthetic pathway.

INTRODUCTION

It has long been considered that aldosterone is formed by the zona glomerulosa from cholesterol through a sequence of enzymatically governed transformations that include, sequentially, cholesterol → pregnenolone → progesterone → deoxycorticosterone → corticosterone → aldosterone. A number of efforts have been made to define the step in this biosynthetic pathway at which angiotensin acts to stimulate the formation of aldosterone. Thus, Brown and associates, using metyrapone to inhibit the conversion of 11-deoxycorticosterone to corticosterone, found that angiotensin stimulated the secretion of deoxycorticosterone and inferred that angiotensin acted on the pre-deoxycorticosterone portion of the aldosterone biosynthetic pathway [1].

In the present study, we have pursued the question still further and have used a new agent, *trilostane*, to inhibit the conversion of pregnenolone to progesterone in an effort to determine whether or not angiotensin stimulates the pre-pregnenolone portion of the aldosterone biosynthetic pathway. Even if angiotensin were found to stimulate the pre-pregnenolone portion of the pathway, this would not exclude the possibility that it might also stimulate late steps, thus enhancing the utilization of later precursors in the formation of aldosterone. Indeed, previous workers have shown that angiotensin stimulates the conversion of radioactively labeled corticosterone to radioactive aldosterone in adrenal cell preparations [2] and in an isolated adrenal mitochondria preparation obtained from dogs treated *in vivo* with angiotensin [3]. In the present study, we have chosen to measure the effect of angiotensin on total aldosterone formation in a system in which the availability of various precursors could be strictly controlled. In order to achieve such conditions, we have added aminoglutethimide to zona glomerulosa cell suspensions to abolish the conversion of cholesterol to pregnenolone [4]. Thus, even if angiotensin were to increase pregnenolone formation in the normal zona glomerulosa, this would be of no consequence in the aminoglutethimide blocked system. Under these conditions, the amount of any particular aldosterone precursor in the system would be determined solely by the amount of exogenous precursor (e.g. pregnenolone) added in any given experiment. In this way the effect of angiotensin on the conversion of pregnenolone to aldosterone could be studied in isolation from the effect of angiotensin on pregnenolone formation.

In short, we wished to study both the early and the late stages in aldosterone biosynthesis in pure zona glomerulosa cell suspensions under conditions in which the early and late steps were functionally

* This study was supported by a grant-in-aid from the National Institutes of Health, USPHS 5-RO1-AMO5318.

† This study was presented in part at the Fifth International Congress of Endocrinology, Hamburg, July, 1976.

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isolated from each other. Such a study has never been performed previously. We have found that angiotensin stimulates aldosterone formation through independent effects on the early phase and on the late phase of aldosterone biosynthesis.

MATERIALS AND METHODS

Bovine adrenal zona glomerulosa cell suspensions were prepared following the method described by Peytremann and co-workers in this laboratory [5]. Using a Stadie-Riggs microtome, outer 0.25 mm slices (including the capsule) were obtained from adrenals of freshly slaughtered calves. The number of adrenals used varied between 10 and 20 per experiment according to their availability. The cells were dispersed by treatment of the slices with trypsin and gentle agitation. The dispersed cells were suspended in Krebs–Ringer bicarbonate buffer containing dextrose 2 g/l, potassium 6 mEq/l, and bovine serum albumen 5 g/l (pH 7.4). Equal aliquots of the cell suspension were then dispensed into 17 × 100 polypropylene tubes. The final vol. in each tube was adjusted to 1 ml by the addition of various treatment agents and/or buffer. Trilostane, aminoglutethimide and angiotensin were each added in 25 μl of a sodium chloride solution (154 mEq/l). Pregnenolone, progesterone and deoxycorticosterone (10 μg) were added in 25 μl of methanol. Each treatment schedule was prepared and subsequently analyzed in duplicate or triplicate. A rack carrying all the tubes was placed in a water bath (37°C) on an automatic shaker for 1 h. The incubation was carried out under 95% oxygen and 5% carbon dioxide. The rack was then placed in a refrigerator and the cell suspension frozen. The entire cell suspension was subsequently thawed and homogenized. Following preparative chromatography the content of pregnenolone, aldosterone and cortisol in the cell suspension was measured using previously described radioimmunoassays [6–8].

Possible contamination of the zona glomerulosa cell suspension with zona fasciculata cells was assessed by measurement of the cortisol concentration in control and angiotensin-stimulated cell suspensions. Cortisol concentrations showed striking increases in approximately 2/3 of the cell suspensions when angiotensin was added; all such preparations were discarded. Only those cell suspensions in which angiotensin stimulated merely small changes in cortisol concentration were used to study the early phase of aldosterone biosynthesis. This was necessary since changes in pregnenolone concentration were used as indices of steroidogenic activity in the early phase of aldosterone biosynthesis, and pregnenolone is a precursor of both aldosterone and cortisol. Therefore, in order to attribute changes in pregnenolone concentration to changes occurring uniquely in the aldosterone producing pathway it was necessary to demonstrate that no major increase in cortisol production had been stimulated by angiotensin.

Trilostane (4α,5-epoxy-17β-hydroxy-3-oxo-5α-androstane-2α-carbononitrile) is a new inhibitor of steroid biosynthesis. Preliminary experiments were performed to examine the effect of trilostane on aldosterone biosynthesis. Aldosterone production was measured in glomerulosa cell suspensions with and without the addition of trilostane (10^{-5}M) under basal conditions and when angiotensin II was added. Trilostane was added to the cell suspensions 15 minutes before angiotensin II. The site of action of trilostane was identified by measuring the conversion of aldosterone precursors to aldosterone in control and in trilostane-treated glomerulosa cell suspensions. Trilostane was supplied by Sterling Wintrop Research Institute.

Aminoglutethimide (3-ethyl-3-(*p*-aminophenyl)-2,6-dioxopiperidine) is an inhibitor of the conversion of cholesterol to pregnenolone [4]. This agent was used to isolate the late phase of the aldosterone-producing pathway. The effect of angiotensin on the late part of the pathway was assessed by comparing the formation of aldosterone from exogenous aldosterone precursors (pregnenolone, progesterone, deoxycorticosterone and corticosterone) in the presence and in the absence of angiotensin, in an aminoglutethimide-treated glomerulosa cell suspension. In this system any aldosterone formed must have come from the added exogenous precursor since endogenous aldosterone biosynthesis was inhibited by treatment with aminoglutethimide. The concentration of aminoglutethimide used in the cell suspensions was 7.6×10^{-4} M. Aminoglutethimide was supplied by the Ciba-Geigy Corporation.

Synthetic Asparaginyl^1-valyl^5 angiotensin II, as the amide, was supplied by the Ciba-Geigy Corporation and used in these studies in a final concentration of 10^{-5} M.

RESULTS

Effect of trilostane on aldosterone biosynthesis

Trilostane inhibited aldosterone biosynthesis in bovine glomerulosa cell suspensions basally and when angiotensin II was added. The results of one experi-

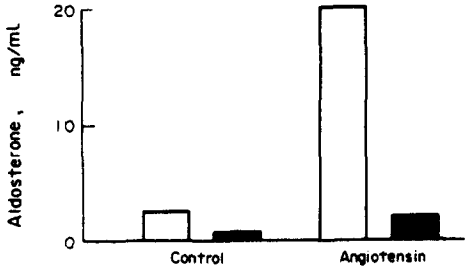

Fig. 1. Effect of trilostane on aldosterone biosynthesis. Comparison of aldosterone concentrations in untreated glomerulosa cell suspensions (open columns) with aldosterone concentrations in trilostane-treated glomerulosa cell suspensions (closed columns), either with or without angiotensin II, indicates that trilostane is a potent inhibitor of aldosterone biosynthesis.

Table 1. Changes in steroid concentrations induced by angiotensin in calf glomerulosa cell suspensions treated with trilostane

Experiment	Steroid measured (ng ml)	Additions to cell suspensions: None	Angiotensin	Trilostane	Angiotensin Trilostane
1.	Aldosterone	1.3	11.0	1.3	1.6
	Pregnenolone	6.5	10.7	29.0	67.0
	Cortisol	3.0	3.6	0.9	0.9
2.	Aldosterone	0.4	11.0	1.0	3.0
	Pregnenolone	2.0	2.5	15.8	63.0
	Cortisol	2.0	3.4	0.8	1.2
3.	Aldosterone	2.5	20.0	0.4	2.0
	Pregnenolone	1.0	1.6	10.4	84.0
	Cortisol	1.2	6.8	0.6	0.8
4.	Aldosterone	1.2	5.2	0.7	0.8
	Pregnenolone	0.2	2.3	13.5	27.6
	Cortisol	1.3	2.6		
5.	Aldosterone	0.9	2.1	0.6	0.6
	Pregnenolone	0.8	0.6	6.8	12.6
	Cortisol	0.5	0.8		
6.	Aldosterone	1.2	6.0	0.6	0.7
	Pregnenolone	1.0	1.7	10.2	29.6
	Cortisol	0.9	1.4		

ment are shown in Fig. 1 and further documentation is provided in Table 1.

The principal site of action of trilostane was shown to be on the conversion of pregnenolone to progesterone. In the presence of trilostane, pregnenolone accumulated in bovine glomerulosa cell suspensions (Table 1). Neither endogenous nor exogenous pregnenolone could be converted to aldosterone whereas the post-pregnenolone precursors, progesterone, deoxycorticosterone, and corticosterone were still readily converted to aldosterone (Fig. 2). These observations indicate that trilostane inhibits the 3β-hydroxysteroid dehydrogenase-4-5-ene-isomerase enzyme system and establish its utility in studying the effect of stimulators on the pre-pregnenolone portion of the steroidogenic pathway.

Effect of angiotensin on the early phase of aldosterone biosynthesis

The detailed findings in 6 experiments are presented in Table 1. When angiotensin alone was added, it stimulated an increase in aldosterone production. When trilostane alone was added to the cell suspensions, pregnenolone accumulated. When angiotensin II was added along with trilostane, the increase in pregnenolone was 2–8 times greater than when trilostane alone was used. These findings indicate that angiotensin stimulates aldosterone formation at a point prior to pregnenolone formation.

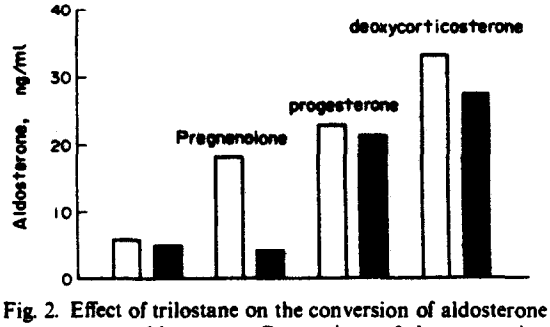

Fig. 2. Effect of trilostane on the conversion of aldosterone precursors to aldosterone. Comparison of the conversion of aldosterone precursors (20 μg) to aldosterone, in untreated glomerulosa cell suspensions (open columns) with that in trilostane-treated glomerulosa cell suspensions (closed columns) indicates that trilostane inhibits only the conversion of pregnenolone to aldosterone ($N = 2$).

Effects of angiotensin on the late phase of aldosterone biosynthesis

The detailed findings in 17 experiments are presented in Table 2. Aldosterone biosynthesis was minimal in the presence of aminoglutethimide and failed to rise when angiotensin II was added. In contrast, aldosterone was readily formed when exogenous aldosterone precursors were added to the aminoglutethimide-treated glomerulosa cells. The total amount of aldosterone derived from deoxycorticosterone was significantly increased when the incubation was performed in the presence of angiotensin ($P < 0.05$). When the percent change induced by angiotensin in the amount of aldosterone derived from deoxycorticosterone is examined, the stimulatory effect of angiotensin becomes more apparent ($P < 0.005$).

DISCUSSION

It has recently been suggested that angiotensin II is less effective in stimulating aldosterone biosynthesis than is angiotensin III, i.e. des-Asp^1-angiotensin II [9]. It is possible that angiotensin II was converted to angiotensin III when it was added to the cell suspension in this study. For this reason, in the discussion of the studies reported here, we have chosen to use the broad term angiotensin, rather than attempting to speculate on the precise form of angiotensin

Table 2. The effect of angiotensin (AII) on the formation of aldosterone (ng) from exogenous aldosterone-precursors (10 μg) added to aminoglutethimide treated bovine glomerulosa cell suspensions

	Additions to cell suspensions											
			Aminoglutethimide		Aminoglutethimide Pregnenolone		Aminoglutethimide Progesterone		Aminoglutethimide Deoxycorticosterone		Aminoglutethimide Corticosterone	
Experiment		AII	—	AII	—	AII	—	AII	—	AII	—	AII
A	2.5	21.0	4.0	1.9	20.0	18.0						
B	< 0.1	3.6	< 0.1	< 0.1	3.4	2.4						
C	< 0.1	6.6	< 0.1	< 0.1	3.5	4.4						
D	0.9	11.0	0.2	0.6	7.0	5.8	8.0	7.2	29.0	50.0	20.0	19.0
E	0.6	2.5	0.8	0.7	1.9	2.5	2.4	1.8	20.0	22.0	3.6	3.2
F	0.2	2.9	0.2	< 0.2	5.8	6.6	7.5	8.2	25.0	28.0	16.0	18.0
G	0.6	2.5	0.7	< 0.2	0.8	1.4	3.2	6.8	22.0	37.0	18.0	19.0
H	0.9	2.1	< 0.3	< 0.3					4.0	4.0	2.5	2.5
I	1.2	6.0	< 1.0	1.0					12.0	12.0	8.0	10.0
J	0.5	2.1	0.6	0.9					2.5	3.0	1.0	1.0
K	0.5	2.4	0.3	0.3					13.0	16.0		
L	0.3	3.3	0.1	0.1					7.3	9.4		
M	0.2	0.7	0.1	0.1					6.8	6.4		
N	0.2	0.5	0.1	0.2					10.0	14.0		
O	0.1	0.5	0.1	0.1					9.3	14.5		
P	0.2	6.9	< 0.1	0.5							8.0	7.6
Q	1.2	5.2	< 0.5	< 0.5							4.5	4.8
Mean Values	0.6	4.7	0.6	0.5	6.1	5.9	5.3	6.0	12.6	16.9	9.1	9.5
Significance of AII effect paired *t*-test		$P < 0.002$		NS		NS		NS		$P < 0.05$		NS

responsible for the stimulation of aldosterone biosynthesis.

Several previous studies to determine the site of action of angiotensin on the biosynthetic pathway leading to aldosterone production have used adrenal cell preparations without attempting to isolate the early and late phases of the pathway [2, 3, 10–12]. Some investigators have demonstrated that angiotensin stimulates the conversion of radioactively labeled cholesterol or acetate to aldosterone [10–12]. Such studies suggested that angiotensin stimulates an early step in aldosterone biosynthesis. However, they did not exclude the possibility of basal conversion of the labeled early phase steroids to late phase precursors whose conversion to aldosterone was then stimulated by angiotensin.

Hunziker and Muller (1977) reported that both basal and serotonin stimulated aldosterone biosynthesis in bisected capsular adrenals are inhibited if the rats from which they are obtained are pretreated with mineralocorticoids for 2 weeks [13]. In contrast, deoxycorticosterone production was increased by pretreatment with mineralocorticoids. These changes in steroidogenesis are compatible with the view that the conversion of deoxycorticosterone to aldosterone is suppressed by pre-treatment with mineralocorticoids. The mineralocorticoids probably exerted these effects by *in vivo* suppression of the renin-angiotensin system; however, angiotensin was not measured. The conversion to aldosterone of radioactively labeled deoxycorticosterone and corticosterone was also impaired in capsular adrenal tissue from mineralocorticoid treated rats. This effect was not seen if the rats also received a sodium deficient diet given to prevent suppression of the renin–angiotensin system. Since enzymatic activity can be induced by increasing substrate availability [2] the impaired activity of the late phase of aldosterone biosynthesis in this study could possibly have occurred because of a chronic decrease in available substrate with consequent "down-regulation" in activity of the late phase enzyme systems. The changes in steroidogenesis reported by Hunziker and Muller were presumably related to suppression of the renin–angiotensin system, but whether angiotensin affected the late phase in aldosterone biosynthesis directly or as a consequence of chronically decreased substrate availability was not examined. Similar criticisms can be applied to other studies which also examined changes in the late phase of aldosterone biosynthesis induced while in continuity with the early phase [2, 3].

In the present study, we have used adrenal inhibitors to isolate various portions of the aldosterone biosynthetic pathway. By using trilostane, we have prevented the adrenal utilization of pregnenolone and therefore, we have been able to use the accumulation of pregnenolone as an index of the activity in the cholesterol to pregnenolone portion of the steroidogenic pathway. This technique has permitted us to demonstrate a direct effect of angiotensin on pregnenolone production in bovine glomerulosa cells. By using aminoglutethimide we have prevented the conversion of cholesterol to pregnenolone and, therefore, have been able to eliminate as an uncontrolled variable the amount of pregnenolone entering the steroidogenic pathway under the influence of angiotensin. This system has permitted us to demonstrate a direct effect of angiotensin on aldosterone biosynthesis at a step subsequent to deoxycorticosterone production and in isolation from its effect on the cholesterol-to-pregnenolone step. Unfortunately, inhibition of the conversion of cholesterol to pregnenolone is not the only effect of aminoglutethimide on the aldosterone biosynthetic pathway. Touitou *et al.* have shown that

this agent also inhibits 18-hydroxylase activity [14]. This enzyme facilitates the conversion of corticosterone to 18-hydroxycorticosterone which is subsequently dehydrogenated to form aldosterone [15]. It seems likely therefore, that the stimulatory effect of angiotensin on the late phase of aldosterone biosynthesis was actually underestimated in the present study.

Although angiotensin enhances the conversion of deoxycorticosterone to aldosterone, from these studies it does not appear that angiotensin influences the conversion of corticosterone to aldosterone (Table 2). It is tempting to postulate that angiotensin stimulates a step in the biosynthetic pathway to aldosterone which is subsequent to deoxycorticosterone production but prior to corticosterone formation. Deoxycorticosterone is hydroxylated in the 11β-position to form corticosterone. Therefore it is possible that angiotensin stimulates the activity of the enzyme, 11β-hydroxylase. However, exogenous corticosterone added to the cell suspensions, may not enter the aldosterone biosynthetic pathway as does endogenous corticosterone. Since exogenous deoxycorticosterone is presumably converted to corticosterone within the glomerulosa cells, it is possible that corticosterone derived from exogenous deoxycorticosterone does enter the aldosterone biosynthetic pathway in a manner similar to endogenous corticosterone. Under these hypothetical circumstances and if endogenous angiotensin stimulates the conversion of only endogenous corticosterone to aldosterone, then in our experiments the effect of angiotensin might be seen when deoxycorticosterone was added but not when corticosterone was added. For these reasons, although we can state with certainty that angiotensin stimulates the aldosterone biosynthetic pathway at a point subsequent to deoxycorticosterone formation, more precise identification of the step at which angiotensin stimulates the late phase of aldosterone biosynthesis is not possible from the present study.

Since deoxycorticosterone is an intermediate in the conversion of both pregnenolone and progesterone to aldosterone, it is of interest to speculate as to why angiotensin did not significantly enhance the conversion of pregnenolone or progesterone to aldosterone while it did facilitate the conversion of deoxycorticosterone. It will be noted in Table 2 that less aldosterone was produced when pregnenolone or progesterone was added to the glomerulosa cell suspensions than when deoxycorticosterone was added even in the absence of angiotensin. It is probable that less deoxycorticosterone was available for conversion to aldosterone when pregnenolone or progesterone was added than when deoxycorticosterone was added. All of the deoxycorticosterone derived from the earlier precursors may have been converted to aldosterone under control conditions. In this situation the enhancing effect of angiotensin on the conversion of deoxycorticosterone to aldosterone would be obscured by the limited availability of deoxycorticosterone.

In the present study we have been able to demonstrate that angiotensin stimulates at least two portions of the aldosterone biosynthetic pathway: the conversion of cholesterol to pregnenolone and the conversion of deoxycorticosterone to aldosterone. Stimulation by angiotensin of more than one step in the aldosterone biosynthetic pathway is analogous to the effects of ACTH on the cortisol biosynthetic pathway noted in recent studies. It has long been established that ACTH stimulates the cholesterol-to-pregnenolone portion of the cortisol biosynthetic pathway. In 1967 Ney and co-workers [16] demonstrated that ACTH prevents the impairment of later steps in the corticosterone biosynthetic pathway that occurs following hypophysectomy in the rat. In 1976, McKenna and co-workers [17] demonstrated that ACTH enhances the formation of cortisol from exogenous pregnenolone, progesterone, 17α-hydroxyprogesterone, and 17α,21-dihydroxyprogesterone in suspensions of cells from the zona fasciculata in which the conversion of cholesterol to pregnenolone has been blocked by aminoglutethimide.

Acknowledgements—The authors wish to acknowledge the technical assistance of Mrs. Rebecca B. Miller. The authors also wish to acknowledge contributions made by Dr. Ronald D. Brown and Dr. Andre Peytremann to the elucidation of the site of action of trilostane.

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