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EFFECT OF ALDOSTERONE ON FLAVIN COENZYME BIOSYNTHESIS IN THE KIDNEY

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SUMMARY

Evidence is presented that aldosterone (2 μg/100 g body weight) induced an increase in [^{14}C]-riboflavin incorporation into flavin adenine dinucleotide (but not flavin mononucleotide) in the kidney of the adrenalectomized rat. Actinomycin D was unable to inhibit this increase in [^{14}C]-riboflavin incorporation into flavin adenine dinucleotide but cycloheximide significantly inhibited this aldosterone-induced increase. Neither metabolic inhibitor had an effect by itself in the absence of aldosterone administration to adrenalectomized rats, even though RNA and protein biosyntheses were inhibited grossly by these agents. Progesterone and corticosterone did not have any effect whereas the synthetic mineralocorticoid, 9α-fluorocortisol, was similar to aldosterone in its action. The aldosterone antagonist, spironolactone, effectively blocked the aldosterone-induced increase of [^{14}C]-riboflavin incorporation into flavin adenine dinucleotide. Again, spironolactone by itself in the absence of aldosterone administration to adrenalectomized rats had no effect. This action of aldosterone on the incorporation of [^{14}C]-riboflavin into flavin adenine dinucleotide was observed in the kidney but not in the liver.

INTRODUCTION

The regulatory role of aldosterone and related mineralocorticoids on active Na^+ transport across a variety of vertebrate epithelial target tissues is well documented[1]. The physiological response to aldosterone in the adrenalectomized animal can be divided into two separable processes *viz.* antinatriuretic and kaliuretic response and this has been reported for the rat[1, 2] and the dog[3, 4]. Edelman and coworkers[1] have implied that the effect of aldosterone might be to increase the activities of some enzymes of the citric acid cycle and the mitochondrial flavoprotein-linked NADH dehydrogenase. Several reports on the enzymic stimulatory property of aldosterone have appeared[5] and it was concluded that the effect of aldosterone was dependent on the increased enzymic activities of the citrate cycle. The work of Fazekas and Sandor[6] showed that in the intact rat, ACTH increased the synthesis of flavin mononucleotide (FMN) in both the liver and kidney but they indicated that it was not known whether this observed effect was due to ACTH *per se* or to the elaborated steroid hormones. It had been shown some time ago that in animal tissues the biosynthesis of flavin coenzymes from riboflavin (RF) proceeded by the sequence:

$$\text{RF} \xrightarrow[\substack{Mg^{2+}\\ATP}]{\substack{\text{Flavokinase}\\(EC\,2.7.1.26)}} \text{FMN} \xrightarrow[\substack{Mg^{2+}\\ATP}]{\substack{\text{FAD Pyrophosphorylase}\\(EC\,2.7.7.2)}} \text{FAD [7, 8]}$$

The implication that aldosterone increased the activity of the mitochondrial flavoprotein-linked NADH dehydrogenase[1] prompted us to investigate the effect of aldosterone on the synthesis of the flavin coenzymes, FMN and flavin adenine dinucleotide (FAD) from RF in the kidney and liver of the adrenalectomized rat. This report deals with the appearance of [^{14}C] label in both the FMN and FAD fractions of the kidney cytosol 60 minutes after a subcutaneous injection of [^{14}C] RF and the differences observed after aldosterone administration to adrenalectomized rats.

EXPERIMENTAL

Methods

Male hooded rats weighing 125–150 g were bilaterally adrenalectomized and ACTH release was suppressed according to the procedure of Kendall *et al.*[9] using dexamethasone. The rats were fed *ad libitum* on Purina rat chow and given drinking water containing 5% dextrose and 1% NaCl. On the third day after adrenalectomy the experiments began.

Expressed in dosages per 100 g body weight, the following were the amounts of steroids and drugs used: Aldosterone, 2 μg; 9 α-fluorocortisol, 2 μg; progesterone, 1·0 mg; corticosterone, 650 μg; spironolactone (SC 9420), 6·5 mg; actinomycin D (AD) 200 μg; cycloheximide (CHI), 200 μg. Four hours after the last injection of dexamethasone (100 μg/100 g body weight), each rat was injected sc with 1 μCi of 2-[^{14}C]-RF (26·1 mCi/mmol) per 100 g body weight with or without the hormones. The metabolic inhibitors, AD and CHI, and the aldosterone antagonist, spironolactone, were administered 30 min before the hormone and [^{14}C] RF, and 1 h after the last injections, the rats were decapitated, the kidneys and a liver lobe removed, and stored frozen until processed.

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One kidney or approximately 500 mg of liver was homogenized in a Potter–Elvehjem type homogenizer with 5 ml of 70% methanol[10]. Protein content of the homogenate was determined by the method of Ehresmann *et al.*[11]; 1·0 ml of the homogenate was centrifuged at 60,000 *g* for 10 min, the clear supernatant transferred into conical tubes containing 6 μg each of "cold" flavin carriers (RF, FMN, FAD) and the mixture evaporated at 50°C in a water bath under a gentle stream of air. The residue was applied to Whatman No. 3 MM chromatography paper (28·5 cm. high) and developed by ascending chromatography for 12 h in the system n-butanol–water–pyridine–glacial acetic acid (3:3:3:1 by vol.)[12]. The three flavin compounds were well separated from each other and were localized under U.V. light.

The radioactivity in the flavin regions was measured by liquid scintillation counting and internal standardization was used for quench correction. The amount of [^{14}C] RF present or incorporated into the flavin compounds was expressed as the corrected c.p.m./mg homogenate protein.

In order to assess the effect of AD and CHI pretreatment on RNA and protein biosynthesis, respectively, the following studies were carried out. Adrenalectomized rats were divided into two groups. One group was pretreated with AD and 30 min later both groups were injected with aldosterone and [^{14}C]-uridine (20 μCi/100 g body weight) to study RNA synthesis. To investigate protein biosynthesis adrenalectomized rats were again divided into two groups; one was pretreated with CHI and then 30 min later both groups were injected with aldosterone and a [^{14}C] amino acid mixture (15 μCi/100 g body weight). The rats in all four groups were decapitated 60 min after the aldosterone and [^{14}C] administration.

The two kidneys of each rat were then homogenized together in 5 vol. of 0·01 M Tris–HCl, pH 7·4. Following homogenization 1·0 ml of each homogenate was used to determine RNA or protein biosynthesis. Aliquots were removed from the homogenates to assay for total RNA and total protein as already described[13]. Also the incorporation of [^{14}C] uridine and [^{14}C] amino acids into total RNA and protein, respectively, were determined[13]. The specific radioactivities of the total RNA and protein were then calculated. The one ml homogenates were next precipitated in the cold with a final concentration of 15% trichloroacetic acid and the radioactivity due to [^{14}C]-uridine or [^{14}C] amino acids, respectively, was measured in an aliquot of the acid-soluble fraction. These radioactivities were also divided by the total amount of RNA or protein, respectively, in order to determine the amount of uptake of [^{14}C]-uridine or-amino acids into the acid-soluble fraction. Since the amount of [^{14}C] associated with RNA or protein precursors, respectively, can influence the specific radioactivities of the total RNA or protein, respectively, these latter specific radioactivities were divided by the appropriate "specific radioactivity" of the acid-soluble fraction. With this measure we were able to arrive at a relatively valid assessment of RNA and protein biosynthesis, respectively.

RESULTS

In the experiments where the rats were not pretreated with dexamethasone (Table 1) aldosterone administration increased the incorporation of [^{14}C] RF only into the kidney FAD fraction by 8·1% over that of the control group ($P < 0·10$). Since Fazekas and Sandor[6] had shown that ACTH increased the synthesis of FMN in the liver and kidney of the intact rat, we decided to suppress the release of ACTH by treating our animals with dexamethasone[9]. This alteration to our procedure gave an increase in the kidney FAD fraction of 18·1% ($P < 0·01$) after aldosterone administration. In the light of these experiments which showed that dexamethasone pre-treatment gave a greater and more significant increase in the incorporation of [^{14}C] RF into FAD, all subsequent studies in this report utilized dexamethasone pretreatment as part of the procedure.

Table 1 also shows that neither progresterone nor corticosterone had the same effect as aldosterone on the incorporation of [^{14}C] RF into FAD in the kidney and were similar to the control group. On the other hand, using the synthetic mineralocorticoid, 9 α-fluorocortisol, no difference was found between this group of rats and those to which aldosterone was administered.

The experiments outlined in Table 2 were designed to demonstrate the influence of AD and CHI pretreatment on RNA and protein biosynthesis, respectively, in the rat kidney. It is seen that AD pre-treatment inhibited RNA biosynthesis by about 50%, whereas CHI pre-treatment abolished about 90% of protein biosynthesis. Thus, the dose levels of these two metabolic inhibitors were appropriate enough to inhibit RNA and protein biosynthesis grossly.

The data in Table 3 demonstrate that AD, an inhibitor of RNA biosynthesis, did not suppress the aldosterone-induced increase in [^{14}C] RF incorporation into FAD in the rat kidney. On the other hand, CHI, an inhibitor of cytoplasmic protein biosynthesis, markedly suppressed this aldosterone-induced increase. Neither metabolic inhibitor had an effect by itself in the absence of aldosterone administration to the adrenalectomized rats. The aldosterone antagonist, spironolactone, effectively blocked the aldosterone-induced increase of [^{14}C] RF incorporation into renal FAD. Again, spironolactone by itself in the absence of aldosterone administration to the adrenalectomized rats had no effect.

The liver in this investigation were obtained from

Table 1. Comparison of the effect of aldosterone and other steroids on flavin coenzyme biosynthesis in rat kidney

Series of groups	Incorporation of [^{14}C] RF into flavin coenzymes (c.p.m./mg homogenate protein)			% Change from control[c]		
	RF	FMN	FAD	RF	FMN	FAD
Control (23)[b]	82 ± 4[a]	51 ± 4	210 ± 9			
Aldosterone (23)[b]	78 ± 4	54 ± 4	227 ± 7	-4.9	+5.9	+8.1*
Control (20)	42 ± 4	37 ± 2	182 ± 9			
Aldosterone (20)	40 ± 4	35 ± 2	215 ± 9	-4.8	-5.4	+18.1**
Control (16)	50 ± 3	40 ± 2	195 ± 10			
Aldosterone (20)	48 ± 3	39 ± 1	230 ± 10	-4.0	-2.5	+17.9**
Progesterone (20)	52 ± 6	41 ± 2	202 ± 9	+4.0	+2.5	+3.6
Control (8)	41 ± 2	44 ± 2	265 ± 13			
Aldosterone (8)	38 ± 3	40 ± 3	316 ± 18	-7.3	-9.1	+19.2***
Corticosterone (12)	43 ± 2	42 ± 2	273 ± 10	+4.9	-4.5	+3.0
Control (8)	28 ± 3	35 ± 2	193 ± 13			
Aldosterone (8)	26 ± 2	33 ± 2	247 ± 12	-7.1	-5.7	+28.0****
9α-Fluorocortisol (8)	30 ± 3	36 ± 2	257 ± 18	+7.1	+2.8	+33.2****

Figures in parentheses are the number of rats used in each subgroup. Each kidney processed separately.

[a] - Mean ± SEM calculated on the basis of the number of rats in each particular subgroup.

[b] - Rats not pretreated with dexamethasone.

[c] - Values calculated on the basis of means of the experimental subgroup against the means obtained for the control subgroup of the same group series. + and - signs indicate an increase or decrease respectively, of the mean values of the experimental subgroups as compared with the control subgroup mean values.

* $p < 0.10$, ** $p < 0.01$, *** $p < 0.025$, **** $p < 0.005$ (unpaired "t" test)

Table 2. Effect of actinomycin D and cycloheximide on RNA and protein biosynthesis, respectively

Series of groups	% Change in RNA biosynthesis due to actinomycin D	% Change in protein biosynthesis due to cycloheximide
Aldosterone (6)		
Aldosterone ± AD (6)	- 48.5 ± 2.3*	
Aldosterone (6)		
Aldosterone ± CHI (6)		- 88.0 ± 2.1**

Refer also to footnote of Table 1.

AD - Actinomycin D; CHI - Cycloheximide.

* $p < 0.005$, ** $p < 0.0005$ (unpaired "t" test).

Table 3. Effect of inhibitors on the aldosterone-induced increase of flavin coenzyme biosynthesis in rat kidney

Series of groups	Incorporation of [^{14}C] RF into flavin coenzymes (c.p.m./mg homogenate protein)			% Change from control		
	RF	FMN	FAD	RF	FMN	FAD
Control (32)	39 ± 2	42 ± 2	270 ± 9			
AD (38)	36 ± 2	41 ± 2	280 ± 8	-7.7	-2.4	+3.7
Aldosterone + AD (37)	36 ± 2	41 ± 2	311 ± 9	-7.7	-2.4	+15.2*
Aldosterone (37)	41 ± 1	43 ± 1	313 ± 10	+5.1	+2.4	+15.9*
Control (17)	35 ± 1	34 ± 1	270 ± 7			
CHI (20)	34 ± 2	33 ± 1	275 ± 7	-2.8	-2.9	+ 1.8
Aldosterone + CHI (20)	37 ± 3	33 ± 1	264 ± 7	+5.7	-2.9	- 2.2
Aldosterone (20)	35 ± 2	35 ± 2	307 ± 7	0.0	+2.9	+13.7*
Control (41)	38 ± 1	39 ± 1	257 ± 6			
SC (34)	37 ± 2	36 ± 2	251 ± 13	-2.6	-7.7	- 2.3
Aldosterone + SC (48)	39 ± 2	37 ± 2	256 ± 8	+2.6	-5.1	- 0.4
Aldosterone (48)	38 ± 2	37 ± 1	306 ± 4	0.0	-5.1	+19.1**

Refer also to footnote of Table 1.

AD - Actinomycin D; CHI - Cycloheximide; SC - Spirolactone.

* $p < 0.0025$, ** $p < 0.0005$ (unpaired "t" test).

the same rats whose kidneys were used to obtain the results in the preceding sections. Although aldosterone induced an increase in the incorporation of [^{14}C] RF into FAD in the kidney, the data in Table 4 show that no such effect was found in the liver.

DISCUSSION

It has been shown that aldosterone increased the incorporation of [^{14}C] RF into FAD in the kidney of the adrenalectomized rat but a greater increment was observed when the rats were pretreated with dexamethasone. Since Fazekas and Sandor[6] had reported that ACTH increased the synthesis of FMN in both the liver (87%) and the kidney (56%), suppression of ACTH release by dexamethasone[9] would in effect remove this stimulus on flavin coenzyme synthesis. Also, dexamethasone would prevent the binding of aldosterone to Type II dexamethasone (glucocorticoid) receptors[14, 15] which would in effect assure us that the influence of aldosterone on [^{14}C] RF incorporation into renal FAD is truly a mineralocorticoid and not a glucocorticoid effect. All our experiments utilized a 60 min *in vivo* labelling period using [^{14}C] RF since it had been shown[10] that the *in vivo* kinetics of the uptake and incorporation of sc injected [^{14}C] RF into kidney flavins of intact rats showed the greatest accumulation of labelled RF after 30 min, FMN at

Table 4. Effect of aldosterone on flavin coenzyme biosynthesis in rat liver

Subgroups	Incorporation of [^{14}C] RF into flavin coenzymes (c.p.m. per mg homogenate protein)			% Change from control		
	RF	FMN	FAD	RF	FMN	FAD
Control (40)	12 ± 1	13 ± 1	121 ± 4			
Aldosterone (40)	12 ± 1	12 ± 1	128 ± 5	0.0	-7.7*	+5.8*

Refer also to footnote of Table I

* Not significant

45 min and FAD at 90 min. These results have been confirmed by us in the adrenalectomized rat.

Our data also show that there was no difference in either the RF or FMN fractions when comparing the control and aldosterone-treated groups and thus suggest that aldosterone had an effect only on the activity of FAD pyrophosphorylase. In the rat liver, Fazekas and Sandor[10] have estimated that K_m of this enzyme and have found it to be significantly lower than the published endogenous FMN concentration, and have suggested that the reaction FMN → FAD is rate limiting. Our data on [^{14}C] RF incorporation into FAD in the liver of these same rats did not show any difference between the aldosterone-treated and control adrenalectomized animals. The apparent lack of similarity in the action of aldosterone on [^{14}C] RF incorporation into FAD in the kidney and liver may possibly be due to the absence of high affinity Type I mineralocorticoid receptors in the liver of adrenalectomized rats[16].

Recently Fazekas and Sandor[17] have reported that in the adrenalectomized rat, aldosterone increased FAD formation in the liver by 82% over control values while FMN biosynthesis was not affected, and that this increase in FAD synthesis was not observed in the kidneys. These results are in contrast to ours possibly because these authors used supramaximal doses of aldosterone (10 μg/day) for a period of 3 days while our experiments were of an acute nature and utilized doses of aldosterone which were more in the physiological range. We also pretreated our rats with dexamethasone which would prevent any binding of aldosterone to Type II dexamethasone receptors[14, 15] and thus assure us that our effect was a mineralocorticoid one and not a glucocorticoid response. Also because of the absence of high affinity Type I mineralocorticoid receptors in the liver of adrenalectomized rats[16], it is quite possible that the response which Fazekas and Sandor[17] observed was a glucocorticoid one.

It has been reported that in the rat the antinatriuretic and kaliuretic responses to aldosterone can be separated[1, 2] and hence might involve independent biochemical pathways. It was shown that AD effectively blocked the antinatriuretic effect, but not the kaliuretic effect of aldosterone in adrenalectomized rats[1]. CHI also inhibited the action of aldosterone on Na^+ transport[1]. Our results demonstrate that AD had no effect on the aldosterone induced increase in [^{14}C] RF incorporation into renal FAD whereas CHI significantly abolished this aldosterone-induced increase. Neither metabolic inhibitor had an effect by itself in the absence of aldosterone administration to the adrenalectomized rats even though RNA and protein biosynthesis were inhibited grossly by these agents.

When progesterone and corticosterone were compared with aldosterone they were found to have no influence on the incorporation of [^{14}C] RF into FAD. However, the potent mineralocorticoid, 9 α-fluorocortisol, was similar to aldosterone in its effect. It seems that the steroid must have potent mineralocorticoid activity (and perhaps have to bind to Type I mineralocorticoid receptors) in order to observe an increase in the incorporation of [^{14}C] RF into renal FAD. Spironolactone, a well known specific antagonist of both the antinatriuretic and kaliuretic responses to aldosterone, effectively blocked the aldosterone-induced increase in the incorporation of [^{14}C] RF into renal FAD. This specific antagonist had no effect by itself in the absence of aldosterone administration to the adrenalectomized rats.

We conclude from these studies that potent mineralocorticoids induced an increase in the incorporation of [^{14}C] RF into FAD in the kidney and that this increase in incorporation might reflect an increase in the biosynthesis of FAD.

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