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THE INFLUENCE OF CORTICOSTEROIDS ON FLAVIN NUCLEOTIDE BIOSYNTHESIS IN RAT LIVER AND KIDNEY*

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SUMMARY

The biosynthesis of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) from exogenous [2-^{14}C]-riboflavin (RF) was investigated in the liver and kidney of intact, adrenalectomized (AX), AX-aldosterone treated, AX-corticosterone acetate treated and AX-cortisol acetate treated rats. Radioactive flavins were determined by a new method using reverse isotope dilution and ion exchange chromatography. It was found that 3 days after adrenalectomy, hepatic FAD formation was reduced by 39% as compared to the intact group. Treatment of AX animals with aldosterone (10 μg/day for 3 days) partially reversed the effect of adrenalectomy (to 82% of the control value). Treatment of AX animals with corticosterone acetate or cortisol acetate decreased hepatic FAD synthesis below the adrenalectomized level. Adrenalectomy and adrenalectomy followed by aldosterone treatment did not affect hepatic FMN formation, while corticosterone or cortisol treatment of AX animals decreased the formation of this flavin nucleotide. No significant changes were observed in renal FMN and FAD biosynthesis by any of the above procedures. These results indicate an extrarenal effect of aldosterone connected to flavoprotein dependent oxidative processes.

It is well known that steroid hormones can influence oxidative processes of different tissues. Glucocorticoids, in pharmacological concentrations, generally decrease oxygen consumption of tissue preparations *in vitro* [1, 2] while aldosterone is believed to increase the rate of mitochondrial oxidation by stimulating the flow of electrons from the reduced nicotinamide nucleotides towards the cytochrome system [3, 4].

Several years ago the effect of corticosteroids was studied on the activity of succinic dehydrogenase in various rat tissues. It was found that physiological doses of aldosterone preferentially stimulated this enzyme in the liver of adrenalectomized rats *in vivo* [5]. Renal succinic dehydrogenase was not affected by the steroid. The observation that aldosterone action is not confined to the kidneys prompted the initiation of the studies described in this paper.

Succinic dehydrogenase is a flavoprotein, containing flavin adenine dinucleotide (FAD) and it is known that the formation and activity of flavoprotein enzymes is largely dependent upon the availability of their coenzymes [6]. In consequence, it was thought that the effects of aldosterone on flavoprotein formation could be studied by monitoring FAD synthesis from exogenous riboflavin (RF).

The biosynthesis of FAD is achieved in two stages in animal tissue. First, RF is converted to flavin mononucleotide (riboflavin-5′-phosphate, FMN) by the enzyme flavokinase. In a second enzymatic step FMN is transformed to FAD in a reaction catalyzed by FAD-pyrophosphorylase [7, 8].

Utilizing recently developed analytical methods [9, 10], first the kinetics of flavin coenzyme biosynthesis in rat liver and kidney were examined *in vivo* and *in vitro* [10]. It was found that 1 h following the subcutaneous injection of [2-^{14}C]-RF, over 80% of the total ^{14}C activity in the liver was present as FAD, while this compound accounted for some 53% of the total renal ^{14}C activity. Thus both organs were found to be very active sites of FAD synthesis [10, 11].

The present study was designed to investigate the effects of aldosterone and corticosterone—two of the major corticosteroids secreted by the rat adrenal—on the biosynthesis of FMN and FAD in rat liver and kidney from exogenous RF.

MATERIALS AND METHODS

Experimental animals. Female Wistar rats weighing 200 g and maintained on Purina rat chow and tap water were used in all experiments. Animals were adrenalectomized by the lumbodorsal approach.

Chemicals. [2-^{14}C]-Riboflavin (S.A. 61 mCi/mmol) was obtained from Amersham Searle, Toronto, Ont. Its homogeneity was controlled by thin-layer chromatography prior to use. Radioinert RF, FMN and

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FAD were obtained from Sigma Chemical Co., St. Louis, Mo. Purification of radioinert flavins and the preparation of carrier solutions was done as previously described [10]. Sephadex preparations were purchased from Pharmacia, Uppsala, Sweden. Steroids utilized in this study were obtained from various commercial sources.

Experimental conditions. The following animal groups were set up: (1) Intact controls; (2) Adrenalectomized (AX) animals with no treatment; (3) AX animals treated with aldosterone (2 × 5 μg daily, administered subcutaneously (sc) in 0·25 ml isotonic saline for 3 days following adrenalectomy); (4) AX animals treated with corticosterone acetate (2 × 5 mg daily, administered intramuscularly for 3 days following adrenalectomy); (5) AX animals treated with cortisol acetate (1 × 10 mg daily, administered intramuscularly for 3 days following adrenalectomy). Animals of all groups were injected with radioactive RF (1·25 or 1·85 μCi/100 g body weight, sc in 1 ml isotonic saline) on the evening of the 3rd postoperative day and sacrificed exactly 60 min later by decapitation and exsanguination. Livers and kidneys were immediately removed and stored at −20°C until analysis. Storage did not exceed 1 week. Under these conditions flavins are stable [10].

Extraction, separation and determination of radioactive flavins in tissue samples. The ^{14}C labeled flavins in liver and kidney tissue were determined by the carrier method utilizing the principle of inverse isotope dilution. The methodology was described in detail previously [9, 10]. Briefly, the flavins were extracted from the tissue samples in the presence of known amounts of radioinert RF, FMN and FAD, separated from each other by column chromatography on DEAE-Sephadex-A-25. Once separated, their S.A. was determined and the total radioactivity incorporated into each flavin calculated. Results were expressed as total disintegration per minute (TDPM) incorporated into the respective flavin per 100 mg tissue/h. For the definition and calculation of TDPM, the reader is referred to a previous publication [10]. Flavins were quantified by spectrophotometry at 450 nm in a Unicam SP 500 spectrophotometer and radioactivity was counted in a liquid scintillation spectrometer (Tri-Carb, Model 3375, Packard Instrument Company, Downers Grove, Illinois).

Results of the studies were analyzed for statistical significance by Student's *t* test.

RESULTS

Effects of adrenalectomy and corticosteroids on the biosynthesis of flavin nucleotides in rat liver

The effects of adrenalectomy and the effects of treating AX animals with aldosterone or corticosterone is shown in Table 1.

Adrenalectomy did not influence the free RF content of the tissue or FMN synthesis. However, incorporation of exogenous RF into FAD was decreased by 39% 3 days after operation. Aldosterone treatment, under these experimental conditions partially restored the adrenalectomy induced FAD biosynthesis deficit (up to 83·6% of the intact value) without influencing FMN synthesis.

Corticosterone treatment of AX animals gave different results. Free RF content and flavokinase activity were both decreased but most significantly, FAD synthesis was reduced by 55% compared to the intact controls and by 16% as compared to the AX non-treated animals.

Effect of adrenalectomy and corticosteroids on the biosynthesis of flavin nucleotides in rat kidney

Results obtained with this tissue are shown in Tables 2 and 3. Data obtained with rat kidney are less clearcut than those with the liver. Adrenalectomy did not influence the renal free RF content or FAD synthesis, while there was a marginally significant increase in FMN synthesis. Aldosterone treatment was without dramatic effect. If anything, aldosterone inhibited marginally FAD synthesis and corticosterone

Table 1. Effects of adrenalectomy and of adrenocortical steroids on the biosynthesis of flavin nucleotides from [2-^{14}C]-RF in rat liver

Experimental animals (N)	RF	%	FMN	%	FAD	%
Intact (10)	1917 ± 245	100	1203 ± 118	100	9556 ± 595	100
Adrenalectomized (14)	1964 ± 167	102·4	1328 ± 110	110·4	5825 ± 288 (P^* < 0·001)	60·9
Adrenalectomized + Aldosterone (10)	2069 ± 295	108	1175 ± 74	97·6	7820 ± 380 (P_x† < 0·001)	81·8
Adrenalectomized + Corticosterone (10)	1400 ± 50	73	1022 ± 16	84·9	4337 ± 217	45·3
	(P_x < 0·01)		(0·01 < P_x < 0·02)		(P_x < 0·001)	

* P: significance of difference from intact animals.

† P_x: significance of difference from adrenalectomized, untreated animals.

Results are expressed as TDPM ± S.E.M./100 mg tissue incorporated 60 min following the sc injection of [2-^{14}C]-RF (1·25 μCi per 100 g body weight).

Table 2. Effect of adrenalectomy and adrenocortical steroids on the biosynthesis of flavin nucleotides from [2-^{14}C]-RF in rat kidney

Experimental animals (no. in group)	RF	%	FMN	%	FAD	%
Controls (10)	9083 ± 1159	100	4074 ± 372	100	14932 ± 592	100
Adrenalectomized (14)	9141 ± 879	100·6	5279 ± 397 ($0{\cdot}05 > P^* > 0{\cdot}02$)	129·5	14809 ± 698	99·1
Adrenalectomized + Aldosterone (10)	9285 ± 1298	102·2	5067 ± 805	124·3	12344 ± 605 ($0{\cdot}02 > P_x > 0{\cdot}01$)	82·6
Adrenalectomized + Corticosterone-acetate (10)	9305 ± 760	102·4	4300 ± 406 ($P_x^* < 0{\cdot}01$)	105·5	13500 ± 504	90·4

* P: significance of difference from intact animals.
† P_x: significance of difference from adrenalectomized, untreated animals.
Results are expressed as TDPM ± S.E.M./100 mg tissue incorporated 60 min following the sc injection of [2-^{14}C]-RF (1·25 μCi per 100 g body weight).

FMN synthesis. Treatment of AX animals with cortisol acetate did not effect the uptake and/or biosynthesis of any of the three flavins in the kidneys.

While cortisol is not a secretory product of the rat adrenal, it is a potent glucocorticoid and some experiments were done along the lines described for aldosterone and corticosterone. Results are shown in Table 3. These animals were injected with a larger amount of radioactive RF and their flavin nucleotide biosynthetic rates were higher in accordance with results published previously [10].

Similarly to corticosterone, cortisol exerted a significant inhibitory effect on both FMN and FAD biosynthesis and the free RF content of the liver was also reduced. The effect of cortisol was more pronounced than that of corticosterone on the free RF content and flavokinase activity.

DISCUSSION

Flavin nucleotide coenzymes are involved in many facets of intermediary metabolism and are coenzymes of a large number of dehydrogenases and oxidases. Their functional group, isoalloxazine undergoes reversible oxidation-reduction and serves as electron donor to the cytochrome system or to direct oxidation reactions involving molecular oxygen. Thus it is logical to suppose that the effects of adrenocorticosteroids upon flavin coenzyme synthesis described in this paper might be connected with tissular oxidation. It is known that adrenalectomy decreases the basal metabolic rate of rats by about 10% [12]. This correlates well with the 40% decrease in hepatic FAD synthesis following adrenalectomy as compared to intact rats (Table 1).

Treatment of rats with cortisone acetate (1–5 mg/day for 6–7 days) was reported to result in a depression of State 3 respiration of liver mitochondria [13, 14]. This effect was explained as a consequence of the synthesis of defective mitochondria [14]. The decrease in hepatic FAD synthesis of rats treated with comparable pharmacological doses of corticosterone might also be connected to an interference of this steroid with mitochondrial oxidative processes.

Table 3. Effect of hydrocortisone-acetate on the biosynthesis of flavin nucleotides in the liver and kidney of adrenalectomized rats

Experimental animals (no. in group)	RF	%	FMN	%	FAD	%
Liver						
Adrenalectomized (5)	1477 ± 53	100	1268 ± 58	100	15019 ± 727	100
Adrenalectomized + Hydrocortisone-acetate (6)	644 ± 58 ($P_x < 0{\cdot}001$)	43·6	521 ± 68 ($P_x < 0{\cdot}001$)	41	9714 ± 818 ($P_x < 0{\cdot}001$)	64·4
Kidneys						
Adrenalectomized (5)	4269 ± 156	100	3941 ± 190	100	25513 ± 990	100
Adrenalectomized + Hydrocortisone-acetate (6)	3864 ± 207	90·5	3431 ± 207 ($P_x > 0{\cdot}1$)	87	27228 ± 1102	106·7

P_x = significance of difference from adrenalectomized.
Results are expressed as d.p.m. ± S.E.M./100 mg tissue incorporated 1 h after the *in vivo* injection of [2-^{14}C]-RF (1·85 μCi/100 g body weight). Adrenalectomized animals were treated for 3 days with hydrocortisone acetate (1 × 10 mg daily, intramuscularly).

Rivlin and Langdon [15, 16] have shown that a non-steroidal hormone, thyroxine has also a profound effect on flavin coenzyme synthesis in rat liver. However, this hormone preferentially stimulates flavokinase activity in the liver of rats made hyperthyroid by T_4 injections. Studies utilizing labeled riboflavin have shown that under these conditions, hepatic FAD synthesis was also increased [17]. Apparently, there is an antagonism between the effects of glucocorticoids and thyroxine at the level of flavin coenzyme biosynthesis. This antagonism might be the molecular basis of the clinically observed beneficial effect of cortisol in thyroid crisis.

An interesting observation, described in this paper is the stimulatory action of aldosterone on the activity of hepatic FAD-pyrophosphorylase in adrenalectomized rats. At present, it is not possible to postulate any theory to explain this effect. However, it seems logical to connect this action of aldosterone with the mitochondrial utilization of FAD. As mentioned previously, aldosterone stimulated succinic-dehydrogenase activity in the liver of adrenalectomized rats [5] and this enzyme is localized in the mitochondria. In addition, it was shown that exogenous aldosterone, added in physiological concentration to liver mitochondria *in vitro* stimulated the State 3 respiration [18]. FAD is very rapidly accumulated by rat liver mitochondria and under the conditions of the present study, 35% of the total radioactive flavin content of the liver was concentrated in the mitochondria, another 35% in the nuclear fraction and the rest equally distributed between the microsomal and soluble fractions [10].

According to present knowledge, both enzymatic steps, leading from RF and FAD through FMN take place in the "soluble" fraction of the cell and once the coenzymes are formed they associate with apoenzymes to form flavo-proteins [6]. This coenzyme—apoenzyme association occurs very rapidly [10] and the excess free FAD is believed to be destroyed by enzymatic hydrolysis [6]. The longterm effect of aldosterone is apparently reflected in both processes. However the possibility of flavoprotein synthesis within the mitochondrion remains open and it could be the subject of future investigations. This cell component is a likely site for direct steroid action taking place within short periods of time.

The effects of aldosterone described in this paper support the theory of Edelman and Fanestil [3] and Fanestil and co-workers [19] which implicates the flavoprotein region of the electron transport chain as the possible site of aldosterone action.

The fact that aldosterone, corticosterone and cortisol hardly influenced renal flavin coenzyme synthesis points to an extrarenal effect of these steroid hormones which is particularly interesting in the context of aldosterone action. Since in these experiments aldosterone was administered in physiological quantities, the stimulation of hepatic FAD-phosphorylase should be an inherent part of the mechanism of action of aldosterone. Whether the action of this mineralocorticoid on flavin synthesis in the liver is connected to the sodium extrusion process remains the subject for further studies.

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