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THE EFFECT OF MELATONIN ON THE BIOSYNTHESIS OF CORTICOSTEROIDS IN BEEF ADRENAL PREPARATIONS *IN VITRO*

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SUMMARY

Melatonin inhibited corticosteroid biosynthesis by various preparations of bovine adrenal cortex *in vitro*. Addition of melatonin to beef adrenal slices (10.8–86.2 nmol/500 mg tissue) inhibited the transformation of carbon-labelled progesterone (2.5 nmol/500 mg tissue) to cortisol (inhibition: 33–63%) and to aldosterone (inhibition: 21–63%). The inhibition was proportional to the amounts of melatonin added though no linear dose–response relationship was obtained. Inhibition of 11β-hydroxylation by adrenal slices and by adrenal mitochondria was only slight. Further experiments with beef adrenal microsomes have shown that melatonin inhibited 17- and 21-hydroxylation. Preliminary kinetic studies seemed to show that melatonin is a non competitive inhibitor of the microsomal 17- and 21-hydroxylases. These observations suggest that melatonin may directly modulate corticosteroidogenesis.

INTRODUCTION

The pineal gland may play some role in the regulation of adrenal cortical function. However, the effect of pinealectomy, pineal hormone(s) and mode of their actions on the metabolism of corticosteroids has not been clearly established [1–4]. It has been reported that in the pinealectomized rats aldosterone and corticosterone secretion rates were significantly elevated [1], suggesting that this increase in secretion might be mediated via ACTH [1, 2]. Another investigator has shown that administration of pineal extracts to rats inhibited aldosterone secretion [5]. Lommer reported that a lipid extract of pineal gland inhibits *in vitro* the 11β-hydroxylase activity of beef adrenals [6]. Decrease in adrenal weights of offsprings of pregnant rats injected with melatonin has also been reported [7]. Gromova *et al.* have shown that subcutaneous injections of melatonin into rats produced increase in corticosterone and simultaneously decrease in aldosterone production [2]. In recent studies Ogle and Kitay have found that corticosterone production and plasma concentrations of pinealectomized rats were significantly higher than that of the control animals [4].

In the present investigation we have attempted to explore the effects *in vitro* of melatonin on the biosynthesis of corticosteroids using the bovine adrenal cortex. Thus the metabolism of [4-^{14}C]-progesterone and [4-^{14}C]-11-deoxycorticosterone by the adrenal cortical slices, mitochondria and microsomes was investigated.

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MATERIALS AND METHODS

Fresh bovine adrenal glands were obtained at the local abattoir and transported to the laboratory in ice-cold saline solution. Following removal of surrounding fat, the glands were carefully dissected, and the tissue was sliced. For cellular fractionation, the slices were homogenized in ice-cold 0.25 M sucrose. Cell fractions were prepared by differential centrifugation in a preparative ultracentrifuge (Model L2-65B Spinco Div. Beckman Instrument, Palo Alto, CA) using a modified version of the procedure described by Schneider and Hogeboom[8]. The mitochondrial sediment was obtained at 15,000 *g* (10 min) and microsomal pellet at 105,000 *g* (60 min).

[4-^{14}C]-Progesterone (S.A.: 55 mCi/mmol) and [4-^{14}C]-11-deoxycorticosterone (DOC) (S.A.: 55 mCi/mmol) were used as substrates. Tritiated aldosterone, cortisol, corticosterone, 17α-hydroxyprogesterone and 11-deoxycorticosterone were used as internal standards. All these radioactive steroids were obtained from New England Nuclear Corporation, Boston, MA and their chromatographic homogeneity was verified prior to their use. Melatonin was purchased from Sigma Chemical Company, St. Louis, MT.

Cortical slices weighing 500 mg or subcellular preparations derived from the same amount of tissue were used in each incubation vessel. These preparations were incubated with the [^{14}C]-substrates (300,000 d.p.m.) in 5 ml of Krebs–Ringer bicarbonate (pH 7.4) supplemented with 11 mM glucose. The subcellular preparations were futher supplemented with 0.48 mM of NADPH. Incubations were carried out in a metabolic shaking incubator at 37° in an O_2 (95%)–CO_2 (5%) atmosphere.

At the end of each incubation, tritiated steroids

Table 1. Effect of various concentrations of melatonin on conversion of [4-^{14}C]-progesterone to cortisol and aldosterone by bovine adrenal cortex

	Amount of melatonin added nmol/ml	% Inhibition of	
		Aldosterone	Cortisol
1.	0	0	0
2.	2.16	21.7	33.9
3.	4.32	50.4	66.8
4.	8.64	40.0	66.1
5.	17.28	63.5	58.6

In all experiments, adrenal cortex slices weighing 500 mg were incubated with [^{14}C]-progesterone (~300,000 d.p.m.). The incubation was carried out at 37° for 3 h. In the absence of melatonin, 11.5% of the substrate was transformed to aldosterone and 15.3% appeared as cortisol.

were added to the media to be used as internal standards. Extraction of the media were carried out with chloroform and ethyl acetate as described elsewhere [9]. The extracts were evaporated to dryness under reduced pressure. For the resolution of extracts, a previously described serial paper chromatographic fractionation scheme was adopted [9–11].

Radioactive zones were detected on paper chromatograms with a radio-chromatogram scanner (Model 7200, Packard Instrument Company). Quantitative measurement of radioactivity was done with a 3-channel liquid scintillation spectrometer (Tri-Carb, Model 3375, Packard Instrument Company). Counting error was kept at ±1% (10^4 net c.p.m. accumulated) and isotope contents were expressed as disintegrations per minute (d.p.m.). Initially the results were calculated as percentage conversion of radioactivity (^{14}C d.p.m.) added as a substrate. The data were corrected for the experimental losses.

Individual metabolites isolated by the chromatographic scheme were further run in suitable chromatographic systems in which their isopolarity with the corresponding radio-inert carrier was established [9–11]. A conversion product was considered pure when constancy of ^{3}H/^{14}C ratios was established in successive chromatographic systems.

RESULTS

Table 1 shows the results obtained from the incubation of bovine adrenal slices with [^{14}C]-progesterone in the presence of various concentrations of melatonin. It can be seen that the conversion to cortisol was inhibited with increasing amounts of melatonin added. The results are expressed as percentage inhibition due to melatonin as compared to the control. In these sets of experiments only cortisol and aldosterone were taken into consideration as these corticosteroids contained most of the steroid hydroxyl groups to be studied in further investigations. At the lower concentrations of melatonin, 2.16 and 4.32 nmol/ml, the percentage inhibition of cortisol was 33.9 and 66.8% respectively. At higher concentrations the effect of melatonin seemed to level off. A similar type of inhibition by melatonin was observed for aldosterone.

To investigate the effect of melatonin on the 11β-hydroxylase, initial studies were conducted on the conversion of [^{14}C]-11-deoxycorticosterone to [^{14}C]-corticosterone by beef adrenal slices and beef adrenal mitochondria. As shown in Table 2, corticosterone formation was inhibited, but not to a significant level.

Further experiments were carried out using adrenal microsomes, as both the 17α- and the 21-hydroxylase are found in this intracellular fraction [12]. Table 3 shows the percentage inhibition in the transformation of [^{14}C]-progesterone to [^{14}C]-17-hydroxyprogesterone and [^{14}C]-11-deoxycorticosterone in the presence of various levels of melatonin. The inhibitory effect of melatonin on the formation on these two steroids was significant and related to the melatonin concentration.

Preliminary experiments to investigate the type of melatonin inhibition seemed to indicate that the inhibition was non competitive. The apparent inhibition

Table 2. Effect of various concentrations of melatonin on the conversion of [4-^{14}C]-11-deoxycorticosterone to corticosterone by bovine adrenal cortex slices and mitochondria.

	Amount of melatonin added nmol/ml	% Inhibition of corticosterone formation	
		Tissue preparation	
		Slices	Mitochondria
1.	0	0	0
2.	2.16	12.7	9.3
3.	4.32	18.9	11.3
4.	8.64	6.7	9.3
5.	17.28	6.7	37.1

Adrenal cortex slices weighing 500 mg were incubated with [^{14}C]-DOC (~300,000 d.p.m.) and incubation was carried out for 3 h. Mitochondria derived from 500 mg of adrenal cortex was incubated with [^{14}C]-DOC (~300,000 d.p.m.) for 30 min in the presence of NADPH (0.48 mM). In the absence of melatonin, 6.6% of the substrate was transformed to corticosterone by slices and 48.5% by the mitochondrial preparation.

Table 3. Effects of various concentrations of melatonin on the conversion of [4-^{14}C]-progesterone to 17α-OH-progesterone and 11-deoxycorticosterone by bovine adrenal cortex microsomes

	Amount of melatonin added nmol/ml	% Inhibition of	
		17α-OH-Progesterone	DOC
1.	0	0	0
2.	2.16	17.9	22.2
3.	4.32	33.9	32.4
4.	8.64	—	38.0
5.	17.28	62.8	75.0
6.	43.20	66.1	86.1

In all experiments, microsomes derived from 500 mg of the adrenal cortex were incubated with [^{14}C]-progesterone (~300,000 d.p.m.) for 30 min in the presence of NADPH (0.48 mM). In the absence of melatonin, 24.2% of the substrate was transformed to 17-hydroxyprogesterone and 10.8% to DOC.

constant (K_i) values for the formation of both 17-hydroxyprogesterone and 11-deoxycorticosterone from progesterone were in the order of 10^{-5} M.

DISCUSSION

The present experiments seem to indicate an inhibitory effect of melatonin on the formation of cortisol and aldosterone from exogenous progesterone by beef adrenal slices. Attempts to pinpoint this effect have shown that the microsomal 17- and 21-hydroxylases were most effected while melatonin interfered only marginally with mitochondrial 11β-hydroxylase.

The mechanism of the above inhibition is not clear. Melatonin failed to give an induced difference spectrum with either mitochondrial or microsomal cytochrome P-450 and available evidence suggests a non competitive inhibition. In addition, one should take into consideration that mammalian adrenals most probably contain two types of steroid 21-hydroxylases with different substrate specificity; one acting upon 17-deoxy substrates while the other hydroxylating preferentially 17-hydroxy precursors [13, 14]. In consequence, there is a good possibility that as far as the inhibition of the "21-hydroxylase" is concerned, the reaction 17-hydroxyprogesterone → cortisol might be affected differently than the reaction progesterone → 11-deoxycorticosterone. In our study, 50% inhibition of both the 17-hydroxylase and the 21-hydroxylase was achieved with a melatonin concentration in the range of 4 nmol/100 mg adrenal tissue preparation (Tables 1 and 3) and the apparent inhibition constant was in the 10^{-5} M range. On the other hand, plasma melatonin concentrations were reported to be in the pmol/ml range in the sheep [15]. However, the adrenal tissue used in this study originated from animals kept under slaughterhouse conditions. It is quite conceivable that the sensitivity of the adrenal cortex to melatonin might change according to different photoperiods. Thus these findings have to be validated on experimental animals kept on controlled light-dark cycles.

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