Effects of angiotensin II and ACTH on normal and tumourous human adrenocortical cells

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Abstract. Isolated adrenocortical cells from 6 patients with a 'normal' zona fasciculata, 4 patients with a 'normal' zona glomerulosa, and tumour cells from 1 adrenocortical adenoma and 1 carcinoma were incubated with and without increasing concentrations of ACTH 1–24 (10^{-13} \text{ M to } 10^{-9} \text{ M}) or Asp^{1}-Ile^{5}-angiotensin II (10^{-11} \text{ M to } 10^{-7} \text{ M}). In 4/5 'normal' cases, cortisol was clearly stimulated by 10^{-13} \text{ M} \text{ ACTH. The maximum of the dose-response curve (5-fold stimulation) was reached at } 10^{-10} \text{ M} \text{ ACTH. Angiotensin II (AII) started to stimulate 'normal' cells at } 10^{-11} \text{ M} \text{ with a maximum (2-fold stimulation) at } 10^{-9} \text{ M} \text{. Aldosterone production by 'normal' cells was less markedly stimulated by ACTH and AII, although the threshold doses for both peptides were similar to those of the cortisol response curves. The cells of the adrenocortical adenoma from a patient with Cushing's syndrome produced large amounts of cortisol and small amounts of aldosterone, both steroids being clearly stimulated by ACTH and AII. The adrenocortical carcinoma cells produced small amounts of cortisol and no aldosterone. Cortisol production responded to ACTH, but not to AII. The results suggest that an activated renin-angiotensin system may stimulate the zona fasciculata, since } 10^{-11} \text{ M AII } (= 10 \text{ pg AII/ml}) \text{ is a normal plasma AII concentration on an unrestricted diet. Clinical evidence supporting this thesis is reviewed. However, cortisol production itself will rarely be increased by AII in vivo, since a down-regulation of ACTH would occur.}

Acute infusion of angiotensin II (AII) into normal man stimulates aldosterone, but not cortisol secretion (Oelker et al. 1974). In hypophysectomized dogs, however, small doses of AII stimulate cortisol secretion (Slater et al. 1963). In vitro experiments with bovine adrenocortical slices (Kaplan 1965) and with isolated human adrenocortical cells (McKenna et al. 1978) also show a stimulatory effect of AII on cortisol production. Furthermore, Vallotton et al. (1981) found AII receptors on bovine zona fasciculata cells with properties different from those of ACTH receptors and of AII receptors of the zona glomerulosa.

Recently, Horner et al. (1979) and Schaison et al. (1980) demonstrated that increased AII levels in patients with adrenal 21-hydroxylase deficiency (whose ACTH levels had been normalized by hydrocortisone substitution) stimulate progesterone, 17-OH-progesterone and 17-hydroxycorticosteroid production. Thus, AII seems to be able to stimulate the zona fasciculata in man. The absence of an effect of AII infusions on plasma cortisol in normal man may either be due to acute counter-regulation of ACTH secretion (Semple et al. 1979) or to large differences between the zona glomerulosa and fasciculata in the sensitivity to AII in short-term experiments.

In order to investigate the latter possibility, we compared dose-response curves of ACTH and AII with respect to in vitro cortisol and aldosterone production by isolated human adrenocortical cells.

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Materials and Methods

Six human adrenal glands (Nos. 1–6) with presumably normal zona fasciculata tissue, and 4 glands with a normal zona glomerulosa (Nos. 1–4) were used. In addition, tissues of an adrenocortical adenoma (No. 7) and of an adrenocortical carcinoma (No. 8) were investigated.

Adrenal glands Nos. 1–4 were obtained from:

1) a 32-year-old male donor of a cadaver kidney, 2) a 70-year-old woman, in whom the left infected kidney (pyonephrosis) had to be removed together with the adrenal gland, 3) a 75-year-old woman with a carcinoma of the right kidney, 4) a 38-year-old male with an adrenal phaeochromocytoma. The adjacent adrenal cortex was used.

Adrenal glands Nos. 5 and 6 were obtained from 2 women (50 and 54 years of age) with primary hyperaldosteronism due to unilateral adrenocortical adenomas. Extratumoural adrenocortical tissue which exhibited no signs of additional micronodular hyperplasia was used exclusively.

No. 7: the adrenocortical adenoma (‘black adenoma’) was removed from a 41-year-old woman with severe Cushing’s syndrome, which was cured by the operation.

No. 8: the large adrenocortical carcinoma was removed from a 35-year-old man with mild Cushing’s syndrome. Cushing’s syndrome was ameliorated by operation, but recurred a few months later before the patient died from metastases.

The adrenal glands were placed into ice-cold normal saline and immediately transferred to the laboratory. Adjacent connective tissue was then removed with scissors, and the tissue was cut into small pieces before it was treated with collagenase and deoxyribonuclease according to Haning et al. (1970) in order to isolate the adrenocortical cells. No attempt was made to separate the zona fasciculata from the glomerulosa. The tissue particles were suspended in Krebs-Ringer-bicarbonate buffer (KRB) containing 2 g/l glucose and 10 g/l bovine serum albumin (Schering, Berlin) to which 0.2 mg/ml deoxyribonuclease from bovine pancreas (Serva, Heidelberg) and 1.5 mg/ml collagenase (Serva, Heidelberg, ‘research grade’) was added. This mixture was incubated in a siliconized glass tube for 45 min at 37°C, while the tissue particles were steadily moved in a stream of a 95% O2:5% CO2 gas mixture. Thereafter, the cells were mechanically dissociated by repeated pipetting and washed three times in cold KRB-buffer containing glucose and albumin. After this procedure, between 80 and 95% of the cells were viable as judged by trypan blue staining.

Incubations for assessing steroid hormone production of the isolated cells were carried out in siliconized round 10 ml ‘Warburg’-vessels. The final incubation volume was 2 ml. Between $9 \times 10^5$ and $4.5 \times 10^5$ cells per vessel were incubated in KR-buffer containing glucose and albumin. The potassium concentration was invariably 4 mm. After 10 to 15 min of pre-incubation at 37°C in a water bath of a type 585 G metabolic shaker (Braun-Melsungen, Germany), 0.1 ml of KRB-buffer (control samples) or the same volumes of a solution of ACTH (1–24β-corticotropin-hexaaceate, Synacthen®, Ciba-Geigy, Wehr/Baden) or AII (Asp1-Ile5-angiotensin II, Schwarz-Mann, Orangeburg, USA) in the same buffer were added to the vessels. During the incubation, the reaction vessels were continuously shaken and gased with 95% O2:5% CO2. All control samples or samples containing a given concentration of ACTH or AII were incubated in duplicate. After 2 h, the incubation was stopped. Tracer amounts of tritiated cortisol and aldosterone (both form Amersham/Buchler, Braunschweig) were added to the cell suspension before labelled and unlabelled steroids were extracted from the medium and the cells with dichloromethane. Steroid separation by paper chromatography and the subsequent quantitation by radioimmunoassay followed the procedure of this

\[ \text{Dose-response curves (means} \pm \text{SEM) of steroid production by ‘normal’ human adrenocortical cells. Cortisol results from patients 1 through 6, aldosterone results from patients 1 through 4.} \text{,} \star \star = P < 0.005 \text{ or} \ < 0.01 \\text{(paired t-test of stimulated vs control steroid production, based on absolute values of Table 1).} \]
Table 1.
Effects of ACTH (upper panel) and angiotensin II (lower panel) on cortisol and aldosterone production by isolated human adrenocortical cells. Figures are means of duplicate incubations.

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Laboratory for the measurement of multiple steroids as described by Schöneshöfer (1977).

All individual data reported are means of duplicate incubations. The paired Student's t-test and 'Geigy-Tablen' were used to assess the significance of differences between steroid production of controls and of cells incubated with ACTH or AII.

Results
1. Cortisol and aldosterone production of 'normal' cells
Since the zona fasciculata cells of the extra-adrenomatous adrenal glands of patients 5 and 6 (Conn's syndrome) can be regarded as 'normal', the data of patients 1 through 6 are combined in Table 1 and Fig. 1. Basal cortisol production varied between 23 and 225 ng/10^5 cells. The adrenocortical cells of the patient with pheochromocytoma produced the lowest amount of cortisol. In 4 of 5 glands that were incubated with 10^{-13} M ACTH, this very small peptide concentration stimulated cortisol unequivocally. The maximum of the dose-response curve is reached at an ACTH concentration of 10^{-10} M (Fig. 1).

The lowest concentration of AII employed (10^{-11} M) stimulated cortisol production in 3 out of 6 cases. The maximum stimulation (unequivocal stimulation in 5 out of 6 cases) occurred at an angiotensin concentration of 10^{-9} M (Fig. 1).

The basal production of aldosterone by 'normal' adrenocortical cells (Nos. 1–4) varied between 0.16 and 1.9 ng/10^5 cells. Again, the cells of the pheochromocytoma patient had the smallest aldosterone production. ACTH stimulated aldosterone production in all cases, but less markedly than cortisol. The 'threshold' dose was 10^{-13} M ACTH in 3 out of 4 cases. Larger doses of ACTH had little additional effect on the mean percentage change of aldosterone output (Fig. 1), although a dose-dependent increase of aldosterone production was observed in cells of patients 1 and 2 (Table 1). AII stimulated aldosterone in the 'normal' cells with a
mean maximum of 200% of control at 10^{-7} M, but in 2 cases the maximum was already reached at 10^{-10} M. Aldosterone production in the extratumoural adrenocortical cells of the 2 patients with Conn's syndrome was very low and not affected by ACTH or AII.

II. Steroid production by tumour cells

Basal cortisol and aldosterone production of the 'black adenoma' was 248 ng/10^5 cells and 60 pg/10^5 cells, respectively (Fig. 2). Thus, cortisol production was slightly higher and aldosterone production markedly lower than in a mixture of 'normal' zona fasciculata and glomerulosa cells. The production of both steroids was unequivocally stimulated in a dose-dependent manner by ACTH as well as by AII.

The basal cortisol production rate of the adrenocortical carcinoma cells (0.34 ng/10^5 cells) was less than 1% of the 'normal' mean (130 ng/10^5 cells), and the aldosterone production rate was close to the sensitivity limit of the radioimmunoassay. Cortisol production was clearly stimulated by ACTH, while the effect on aldosterone was equivocal. AII stimulated neither cortisol nor aldosterone production.

Discussion

The adrenocortical cells obtained from patients 1 through 6 were mostly from the zona fasciculata, since the glomerulosa is a very thin layer in man. Nevertheless, aldosterone was well measurable in unstimulated incubates of adrenal cells from patients 1 through 4, whose zona glomerulosa could be expected to be 'normal'. Aldosterone production by extratumoural cells of patients 5 and 6 (aldosterone-producing adenoma) was low and not stimulated by ACTH or AII. This is probably due...
to prolonged renin suppression in these patients. Cortisol production by the cell suspensions was of such an extent that the eluates after paper chromatography had to be greatly diluted prior to radioimmunoassay.

We do not know whether the in vitro steroid production by adrenocortical cells is influenced by the special circumstances of anaesthesia and operation, and of prolonged brain death in patient No. 1. It is also not known to what extent separation of cells by the manoeuvre described changes cell membrane receptors or other morphological or biochemical aspects of the cells. With this proviso in mind, we are impressed by the extraordinary sensitivity of human fasciculata cells to ACTH. 10^{-13} \text{M} ACTH (about 0.3 pg ACTH 1–24/ml incubate) clearly stimulated cortisol production in 4 out of 5 cell preparations. Fig. 1 shows that maximum stimulation was reached at 10^{-9} \text{M} (about 3 ng ACTH 1–24/ml incubate). In accordance with a single observation by McKenna et al. (1978) in fasciculata cells from a patient with Cushing’s disease, we found that AII starts to stimulate cortisol secretion at 10^{-11} \text{M}. The maximum of the dose-response curve is reached at AII concentrations between 10^{-9} \text{M} and 10^{-8} \text{M}.

Aldosterone is also clearly stimulated by 10^{-13} \text{M} of ACTH 1–24 with a rather flat plateau between 10^{-13} \text{M} and 10^{-10} \text{M} of ACTH. Stimulation of aldosterone in ‘normal’ adrenocortical cells by AII is less pronounced than with ACTH, and even less marked than cortisol stimulation by AII. Neither ACTH nor AII stimulated aldosterone production by extratumoural adrenocortical cells of patients 5 and 6 (Conn’s syndrome) in accordance with findings of Brown et al. (1980) who explained this irregularity with a reduced number of AII receptors. Since the glomerulosa cells were probably greatly outnumbered by fasciculata cells, and since we do not know whether high concentrations of fasciculata steroids in the environment inhibit aldosterone production (Newton & Laragh 1968; Biglieri et al. 1969), we restrict the further discussion to cortisol.

10^{-11} \text{M} AII (threshold dose for cortisol stimulation in vitro) is about 10 pg/ml AII. This would be a normal plasma concentration in man on an unrestricted salt diet (Oelkers et al. 1974, 1975, 1978). After 4 days on a low salt diet (10 to 20 mM sodium per day), plasma AII levels rise to 40 to 80 pg/ml. However, cortisol levels are unaffected, and so they are during acute or chronic infusions of AII into normal man with an increase of plasma AII levels to between 50 and 300 pg/ml (Oelkers et al. 1974, 1978). It is possible that cortisol stimulation during AII infusion is immediately counterbalanced by a fall in plasma ACTH concentration. Semple et al. (1979) observed an acute fall in plasma ACTH (measured by an extremely sensitive cytochemical assay) during AII infusion (approximately 2 ng/kg/min) but failed to see the expected transient rise in plasma cortisol. Thus, the possibility exists that AII lowers plasma ACTH independent of cortisol. Clinical evidence also favours an effect of AII on the zona fasciculata, which seems to be masked by a down-regulation of ACTH secretion:

Cade et al. (1967) observed that, in most patients with renal artery stenosis and hypertension, a circadian rhythm of plasma cortisol was absent. The rhythm was normalized after successful repair of the renal artery. David et al. (1972) found abnormalities of cortisol secretion in 2 children with aldosterone deficiency and salt wasting. On a low sodium diet (renin and AII probably greatly increased), cortisol production rate was incompletely suppressed by dexamethasone, while suppression was normal after mineralocorticoid and salt substitution. Similar observations were made by Horner et al. (1979) and by Schaison et al (1980) in patients with the simple virilizing form or the salt-losing form of adrenal 21-hydroxylase deficiency: in spite of normalized ACTH levels due to glucocorticoid substitution, plasma renin and 17-OH-progesterone levels were markedly increased. Administration of fludrocortisone normalized both renin and 17-OH-progesterone.

Under the conditions mentioned above, the renin-angiotensin system is chronically activated. This may augment the influence of AII on the zona fasciculata. Recently, we re-evaluated the data of our prolonged AII infusion experiments in sodium replete and deplete man. While plasma cortisol levels at 09.00 h were slightly decreased during prolonged AII infusions in sodium replete subjects, a slight, significant increase occurred in others who had been on a low sodium diet for several days (Schöneshöfer & Oelkers 1980).

Provided the experimental procedure employed does not lead to major changes in the properties of AII receptors, the data of the present study suggest that the effect of AII on the zona fasciculata is minimal in the range of normal plasma AII levels, but may become significant once the renin-angiotensin system is activated. AII seems to affect early
steps of steroid biosynthesis, since its infusion leads to an increase in 17-OH-progesterone levels in certain conditions (Schaison et al. 1980). This mechanism of action may be shared with the zona glomerulosa, in which AIH seems to exert an additional effect on late biosynthetic steps (Fraser et al. 1979). The effect of AIH on the zona fasciculata will rarely lead to a clear-cut rise in plasma cortisol levels, since a fall in plasma ACTH might occur. Thus, ACTH remains the dominant regulator of cortisol secretion in spite of a stimulatory effect of AIH on the zona fasciculata.

The piece of tissue taken from the ‘black adenoma’ of patient 7 was not adjacent to the tumour capsule. Therefore, it probably did not enclose ‘normal’ zona glomerulosa particles. It appears that the tumour itself contained the cells that produced both cortisol and aldosterone. It is unknown whether the two steroids were produced by the same cells or by different cells. The production of both steroids was significantly stimulated by ACTH and AIH in this benign tumour. Concomitant production of aldosterone and cortisol by an adrenal adenoma has been reported previously by Komiya et al. (1979). However, that patient presented with Conn’s syndrome, while in our patient, Cushing’s syndrome was the clinical diagnosis. Unfortunately, urinary or plasma aldosterone were not measured before operation.

Patient No. 8 had a metastasizing adrenocortical carcinoma. The very large primary tumour had led to a mild Cushing’s syndrome. Correspondingly, the basal cortisol production rate of the tumour cells in vitro was extremely low; it was clearly stimulated by ACTH. This tumour contained only traces of aldosterone (if any), and no increase in aldosterone production by ACTH or AIH was observed.

The results of our experiments, taken together, suggest that in vitro incubation of human adrenocortical cells is a useful complementary method for studying physiological and pathophysiological problems, provided the results are cautiously interpreted.

References


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