Intra-adrenal factors are not involved in the differential control of cortisol and adrenal androgens in human adrenals

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Abstract

The differential control of adrenal androgens and cortisol may be due to intra-adrenal factors, which may be age- or sex-related, or due to extra-adrenal factors, such as circulating hormones. The purpose of this study was to identify any intrinsic differences that may exist in steroidogenic production occurring within adrenals obtained from males and females, and any maturational differences that may evolve with age. Using human adrenals from 48 transplant donors (32 males, 16 females; ages 5–60 years), the influences of age and sex on basal production of and ACTH-stimulated cortisol, androstenedione and dehydroepiandrosterone (DHEA) were examined in freshly prepared adrenal cell suspensions. Basal and ACTH-stimulated cortisol, androstenedione and DHEA production were similar in adrenals from males and females and did not correlate significantly with age when the whole group was examined. When steroidogenesis in male and female adrenals was examined separately against age, a significant correlation was observed only for basal and ACTH-stimulated androstenedione in adrenals from males in the younger age group, 5–30 years (basal: $r = 0.84$, $P = 0.0001$; ACTH-stimulated: $r = 0.52$, $P = 0.007$). Examination of the relationships between the steroids disclosed that the basal and ACTH-stimulated cortisol/androgen ratios did not correlate significantly with age, but the androstenedione/DHEA ratio showed a significant direct relationship with age in males only (basal: $r = 0.53$, $P = 0.006$; ACTH-stimulated: $r = 0.5$, $P = 0.01$).

These data suggest that the influences of sex and age are minor in the modulation of adrenal steroidogenesis and support the concept that extra-adrenal factors dominate in the differential modulation of adrenal androgens and cortisol. The relationship between the androstenedione/DHEA ratio and increasing age in men is consistent with the recently reported stimulatory effect of testosterone on adrenal steroidogenesis by induction of the conversion of DHEA to androstenedione.

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Introduction

Whereas it is well established that cortisol production is under the control of adrenocorticotrophic hormone (ACTH), it has been known for some time that adrenal androgens are regulated in a more complex manner (1). Adrenal androgens are controlled partly, but not exclusively, by ACTH. Several physiological and pathological situations exist in which there is a divergence of adrenal androgen and cortisol secretion (1–3). Cortisol concentrations are increased in stress and anorexia nervosa, whereas androgen concentrations are suppressed (4). In some patients with partial hypopituitarism, cortisol concentrations are normal and androgen concentrations are suppressed (5). Patients with ectopic ACTH syndrome have increased cortisol concentrations, whereas androgen concentrations are increased, normal or decreased (6). Circulating adrenal androgens demonstrate a striking and unexplained relationship with age. Adrenarche is characterized by an increase in blood concentrations of adrenal androgens

at the end of the first decade of life. Serum concentrations of adrenal androgens peak at 20–30 years of age and remain at this level throughout the 4th and 5th decade. With ageing, the concentration of adrenal androgens and their response to ACTH decline (7, 8). This pattern of adrenal androgen concentrations is seen in both sexes (9) and differs from that of adrenal glucocorticoid, as serum cortisol concentrations and cortisol responsiveness to ACTH remain relatively constant throughout life (8). When ACTH is administered to hypophysectomized chimpanzees, it is adequate to maintain cortisol production but, given alone, it is unable to maintain a normal cortisol/androgen ratio (10). Therefore the evidence suggests that some non-ACTH mechanism, either alone or in combination with ACTH, is involved in the control of adrenal androgens (5).

The control of adrenal androgens may be through the agency of 'intra-adrenal' or 'extra-adrenal' factors. The potential endogenous adrenal gland mechanisms regulating adrenal androgen secretion include maturation and zonation of the adrenal gland (11), adrenal

blood flow that may expose the inner zone to high concentrations of cortisol (12), and changes in the properties of the adrenal enzymes and their co-factors (13–15). Alternatively, factors outside the adrenal gland have been proposed, such as prolactin (16), growth hormone (17), insulin (18), sex steroids (19, 20) and fragments of the ACTH precursor molecule, pro-opiomelanocortin, such as β -endorphin (21) and joining peptide (21, 22). The existence of such a cortical androgen stimulating hormone has been disputed (23–25). The present study was designed to examine the influence of ageing and sex on the relative production of cortisol, androstenedione and dehydroepiandrosterone (DHEA), basally and in response to ACTH, and thus enable us to examine the influence of possible endogenous maturational changes that may influence differential production of cortisol and androgens.

Materials and methods

Subjects

Human adrenals were obtained at the time of kidney resection from 48 brain dead transplant donors, 32 males aged 28.8 ± 11.3 years and 16 females aged 37.3 ± 21.7 years (mean $\pm$ s.d.). The large majority of the donors had suffered subarachnoid haemorrhage or head injuries. Before kidney resection, some donors had been pretreated with dexamethasone and desmopressin (DDAVP) or with dopamine and antibiotics. None of the donors had chronic underlying disease that might be expected to influence steroidogenesis. They ranged in age from 5 to 60 years. The study protocol was approved by the Ethics Committee of St Vincent's Hospital.

Cell suspension protocol

The adrenal cell suspension was prepared at $0-4^\circ\text{C}$. Excess fat and connective tissue were removed and the adrenals were chopped into 1 mm cubes. No attempt was made in these studies to separate cells of the zonae glomerulosa, fasciculata and reticularis. This tissue was incubated in Eagle's modified essential medium (EMEM, Flow Labs, Scotland, 10.105–22) containing collagenase (Sigma, Poole, Dorset, UK, C0130) 2 mg/ml, for 20 min in a shaking water-bath at 37°C . After centrifugation at 400 *g* for 15 min, the supernatant was discarded. The pellet was resuspended in EMEM containing 0.2% BSA, and cells were physically dispersed by passage up and down teflon tubing attached to a syringe. This process was repeated three times and dispersed cells were filtered, combined and centrifuged at 400 *g* for 15 min. The resultant pellet of adrenal cells was washed in EMEM and centrifuged before final resuspension in EMEM containing BSA 0.5%, calcium 6.4 mmol/l, and vitamin C 1 mmol/l.

The cells were examined microscopically; the cell count in ethidium bromide acridine orange obtained using a haemocytometer demonstrated that $87 \pm 12.5\%$ (mean $\pm$ s.d.) of the cells were viable. Three different cell types were observed. The zona glomerulosa cells were identified as small spherical cells containing lipid droplets, and the larger spherical cells were identified as arising from the zona reticularis. Cells from the zona fasciculata were recognized by their irregular shape and abundant lipid droplet content; these cells comprised approximately 50% of the adrenal cell count. Aliquots of cell suspension, 800 μl , were added to plastic culture tubes (NUNC 0–43141) that contained 100 μl EMEM (basal) or ACTH(1–39) (Peninsula Laboratories, Liverpool, England). The final volume was made up to 1 ml with EMEM. The tubes containing cells and additions were incubated in a shaking water-bath under 100% oxygen for 2 h at 37°C . Cells were prepared on 48 separate occasions from 48 pairs of human adrenals. On each occasion, cells were incubated in the absence of ACTH (basal conditions) and in the presence of ACTH 10^{-7} mol/l (ACTH-stimulated conditions) in three to five replicate tubes. When steroid production was assessed in these replicate tubes, the variability was usually less than 15%. If large discrepancies occurred, the results of the assay were rejected. Preincubation tubes that contained cells without any added peptide were removed before incubation and stored at 4°C . At the end of incubation, the cell suspensions were frozen and the intracellular steroids were released into the medium by repeated freezing and thawing. The steroids were measured by specific radioimmunoassay. The protocol was similar to that previously used for guinea-pig adrenal cells (26). The cells were capable of responding to ACTH with a significant increase in production of cortisol, androstenedione and DHEA. In order to standardize steroid production from one cell preparation to another, the values obtained were adjusted for the number of cells present by expressing steroid production as pmol/ 10^4 cells.

Steroid measurement

Cortisol was measured by direct radioimmunoassay using antiserum supplied by Guildhay (Guildford, Surrey, England), cortisol-3-(*O*-carboxymethyl)-oximo [2- ^{125}I]-iodohistamine (Amersham Int., Amersham, England) and phosphate buffer 0.5 mol/l, pH 7.4. Aliquots of cell lysates and standards were incubated in the presence of antibody and radiolabelled cortisol overnight at 4°C . Unbound cortisol was separated from antibody-bound cortisol using 1% dextran–gelatin-coated charcoal, followed by centrifugation at 3100 r.p.m. for 15 min. The supernatant was decanted and the radioactivity in the free fraction (the charcoal pellet), was measured using a gamma counter.

Androstenedione and DHEA concentrations were

measured by radioimmunoassay after extraction of steroids with diethyl ether and further purification by chromatography over celite (27). The androstenedione assay used androstenedione-10- $[^{125}\text{I}]$ -iodine derivative (Diagnostic Products UK Ltd, Abingdon, Oxfordshire, England) as the radiolabel and the antiserum was obtained from Radioassay Systems Labs (RSL, Carson, USA; Lot No. 1440 R1/P). The DHEA assay used dehydro-[1, 2, 6, 7- ^3H]-epiandrosterone, (Amersham; code TRK 157) as radiolabel; the antiserum was obtained from ICN Biomedicals Inc. (Diagnostics Division, Costa Mesa, CA 92629, USA, Lot R1-46-21). The specificities, sensitivities and precision of the assays have been reported previously by us (26).

To identify changes in the production of the steroids relative one to another, we utilized androstenedione/cortisol, DHEA/cortisol and androstenedione/DHEA ratios in both basal and ACTH-stimulated cell suspensions.

Statistical analysis

Results are reported as mean $\pm$ s.e. Steroid production in the male and female groups was compared using the Mann-Whitney *U* non-parametric test. The correlation of each group with age was assessed by using regression analysis. A *P* value < 0.05 was considered to be statistically significant.

Results

Cortisol and androgen production in human adrenals in vitro

Cortisol, androstenedione and DHEA production was similar in cells from adrenal glands obtained from males and females under basal or ACTH-stimulated conditions (Fig. 1). However, when the basal and ACTH-stimulated androstenedione/cortisol, DHEA/cortisol and androstenedione/DHEA ratios were compared between males and females, the ACTH-stimulated androstenedione/cortisol ratio (male 0.31 ± 0.13 , female 0.07 ± 0.06 ; $P < 0.05$) and androstenedione/DHEA ratio (male 2.7 ± 0.05 , female 1.5 ± 0.88 ; $P < 0.05$) were significantly greater in the males. When basal and ACTH-stimulated cortisol, androstenedione and DHEA production was examined against age in the total group, no significant correlation was observed (Table 1). However, analysis of values obtained with male and female adrenals separately revealed that, in the male glands, basal and ACTH-stimulated androstenedione concentrations correlated significantly with age ($r = 0.41$, $P = 0.02$; $r = 0.47$, $P = 0.007$; Fig. 2), although cortisol or DHEA did not correlate significantly with age in the group (Table 1). Basal and ACTH-stimulated cortisol, androstenedione and DHEA did not significantly correlate with age in the female group (Table 1). When the male group was divided into almost

Figure 1 Comparison of basal and ACTH-stimulated cortisol, androstenedione and DHEA production in female (open bars) and male (solid bars) human adrenal cells *in vitro*. Results are expressed as the mean $\pm$ s.e. of values obtained in 32 male and 16 female human adrenal cell suspensions. Adrenal cells responded to ACTH by producing a significant increase in cortisol, androstenedione and DHEA synthesis. However, there were no significant differences (NS) in production of these steroids between male and female cells under basal or ACTH-stimulated conditions.

Table 1 Correlation coefficients for basal and ACTH-stimulated cortisol, androstenedione and DHEA production with age (years) in human adrenal cells.

Group	Correlation coefficient (<i>r</i>)					
	Cortisol		Androstenedione		DHEA	
	Basal	ACTH-stimulated	Basal	ACTH-stimulated	Basal	ACTH-stimulated
All	0.23	0.21	0.22	0.31	0.14	0.12
Male	0.36	0.189	0.41*	0.47**	0.011	0.074
Female	0.114	0.346	0.135	0.114	0.48	0.382

Significant correlations: * $P = 0.02$, ** $P = 0.007$.

equal numbers of specimens from younger (less than 30 years, $n = 17$) and older (30 years and over, $n = 15$) individuals, it was observed that the significant correlation of androstenedione with age was confined to the younger subgroup (basal: $r = 0.84$, $P = 0.0001$; ACTH-stimulated: $r = 0.50$, $P = 0.0089$; Fig. 3). No significant correlation was observed when androstenedione was examined against age in adrenals from subjects aged 30–60 years (basal: $r = 0.29$, $P = 0.36$, ACTH-stimulated: $r = 0.21$, $P = 0.417$).

The possible effects of pretreatment of the donors on the subsequent *in vitro* steroidogenesis of adrenal cells was examined by subdividing the donors for whom adequate information was available into three groups: those treated with dexamethasone and DDAVP ($n = 14$), those treated with dopamine and antibiotics ($n = 15$) and those who received no pretreatment ($n = 7$). There were no significant differences in basal or ACTH-stimulated cortisol, androstenedione or DHEA production by adrenal cells between the three groups

of donors. Furthermore, there were no significant correlations of steroid production with age in any of the three groups.

Cortisol, androstenedione and DHEA interrelationships

In the male group, basal and ACTH-stimulated androstenedione/DHEA ratios correlated significantly with age ($r = 0.53$, $P = 0.006$; $r = 0.50$, $P = 0.001$ respectively; Fig. 4), but the DHEA/cortisol or androstenedione/cortisol ratios did not (Table 2). In the female adrenals, the basal and ACTH-stimulated androstenedione/DHEA, DHEA/cortisol and androstenedione/cortisol ratios all failed to correlate significantly with age (Table 2). When the androstenedione/DHEA ratio in males was examined for the two age categories (5–30 years and 30 years and over), the basal androstenedione/DHEA ratio in the younger group correlated significantly with age ($r = 0.6$, $P = 0.0175$), but the ACTH-stimulated androstenedione/

Figure 2 Basal and ACTH-stimulated androstenedione production plotted against age in male human adrenal cells *in vitro* ($n = 32$). There was a statistically significant correlation of both basal (correlation coefficient $r = 0.41$, $P = 0.02$) and ACTH-stimulated androstenedione production ($r = 0.47$, $P = 0.007$) with age in male adrenal cells which was not seen in female adrenal cells.

Figure 3 Basal and ACTH-stimulated androstenedione production plotted against age ranging from 5–30 years, in male human adrenal cells *in vitro* ($n=17$). There was a statistically significant correlation of both basal (correlation coefficient $r=0.84$, $P=0.0001$) and ACTH-stimulated androstenedione production ($r=0.50$, $P=0.0089$) with age in adrenal cells from younger males.

DHEA in the younger group and basal and ACTH-stimulated androstenedione/DHEA in the older group did not correlate with age.

Discussion

Some authors have suggested that the increase in adrenal androgen production at adrenarche is due to

some maturational change within the adrenal gland itself (11–13). It has been reported that the zona reticularis, the innermost zone of the adrenal cortex that predominantly secretes adrenal androgens, appears at the time of adrenarche (11). However, no reciprocal decrease in the size of the zona reticularis occurs during the 5th decade of life, when the adrenal androgen concentrations begin to decline. Another

Figure 4 Basal and ACTH-stimulated androstenedione (Andro)/DHEA ratio plotted against age in male human adrenal cells *in vitro* ($n=32$). There was a statistically significant correlation of both basal (correlation coefficient $r=0.53$, $P=0.006$) and ACTH-stimulated androstenedione/DHEA ratio ($r=0.50$, $P=0.001$) with age in male adrenal cells which was not seen in female adrenal cells.

Table 2 Correlation coefficients for the relationship of basal and ACTH-stimulated androstenedione (Andro)/DHEA, DHEA/cortisol and androstenedione/cortisol ratios with age (years) in male and female human adrenal cells.

Group	Correlation coefficient (<i>r</i>)					
	Andro/DHEA		Andro/cortisol		DHEA/cortisol	
	Basal	ACTH-stimulated	Basal	ACTH-stimulated	Basal	ACTH-stimulated
Male	0.53*	0.50**	0.383	0.355	0.23	0.199
Female	0.297	0.269	0.253	0.022	0.46	0.04

Significant correlations: ** $P = 0.001$, * $P = 0.006$.

possible mechanism is that of long-term exposure of the zona reticularis to cortisol as a consequence of blood flow through the adrenal from the outer cortex to the inner, which may bring about an alteration in the enzymes involved in adrenal androgen production (23). It has also been suggested that maturational changes in the activities of some adrenal enzymes (17-hydroxylase, 17-20-lyase and the 3β -hydroxysteroid dehydrogenase, Δ^{4-5} isomerase system) may account for alterations in steroidogenesis favouring androgens (13–15). Alternatively, factors extrinsic to the adrenal may specifically control adrenal androgen biosynthesis and secretion – for example, the putative cortical androgen stimulating hormone. Several studies support this concept. Using our human adrenal cell preparations, we have reported that joining peptide and β -endorphin, peptides arising from the ACTH precursor pro-opiomelanocortin, have androgen-stimulating properties (21). Parker *et al.* (22) also reported stimulatory effects of joining peptide on adrenal androgen production, but others have not confirmed this (24, 25). Evidence also exists that gonadal steroids influence adrenal glucocorticoid and androgen secretion: for example, testosterone increases the adrenal response to ACTH stimulation (19, 20). A 60 000 molecular weight glycoprotein isolated from human pituitary has been reported to stimulate DHEA but not cortisol secretion (28), and another study has shown that a peptide other than ACTH from human fetal pituitary stimulated DHEAS but not cortisol, in fetal adrenal cells (29).

The present study was undertaken to identify intrinsic differences that may exist in steroidogenesis occurring within adrenal glands from males and females and differences that may evolve with age. We used short-term incubations of freshly prepared human adrenal cells, as these are more likely to reflect *in vivo* conditions than are cell cultures. Our findings suggest that sex differences and maturational changes of the adrenal gland have only a minor influence in the modulation of adrenal androgens. The only significant relationship with age was observed in young men, and this was confined to androstenedione. We found no significant correlations between age and cortisol or DHEA in male or female adrenal cells, or between age and androstenedione in females or older males. Thus changes in female adrenal androgen production at

adrenarche are unlikely to be due to intra-adrenal factors. The absence of any relationship of steroid concentrations with age in women and older men suggests that specific circumstances operate in young men to influence adrenal steroidogenesis. This is consistent with observations that testosterone affects adrenal steroidogenesis by increasing the response of the adrenal androgens to ACTH stimulation (20). Thus the significant correlation observed between androstenedione and age in our young male group may be due to exposure of the adrenal gland *in vivo* to increasing testosterone concentrations, which occurs in the younger group. However, the mechanism by which testosterone may affect adrenal steroidogenesis has not been defined previously. Oestrogens have been shown to inhibit DHEA production (30), and evidence from clinical studies suggests that androgens may inhibit 11β -hydroxylase and 21 -hydroxylase enzyme activity (19, 31). Our data that suggest a direct correlation of age with increasing androstenedione/DHEA ratios are consistent with increasing conversion of DHEA to androstenedione with age in adrenals from males younger than 30 years, as a result of increased 3β -hydroxysteroid-dehydrogenase, Δ^{4-5} isomerase enzyme system activity. The normal increase in blood concentrations of testosterone from testicular origin in young men appears to enhance adrenal conversion of DHEA to androstenedione.

While it is theoretically possible that the pretreatment of the donors might effect subsequent steroidogenesis in the adrenal cells, we observed no differences between cortisol or androgen production in adrenal cells from donors who had no pretreatment and those pretreated with dexamethasone and DDAVP or dopamine and antibiotics. The lack of any effect of donor pretreatment on subsequent steroidogenesis suggests that any such influence on the adrenal is mediated via an acute extra-adrenal mechanism. Furthermore, the specific steroidogenic pattern seen in the adrenals from young men only was not obscured either by stress or by pretreatment of donors. Thus the model used provides useful new insights into the differential control of adrenal glucocorticoid and androgen production.

Adrenal androgen production did not correlate with age or sex, with the exception of androstenedione in

younger male adrenals, suggesting that factors exogenous to the adrenal gland are more important in the control of adrenal androgens than are intrinsic changes. Indeed, it is possible that the correlation of androstenedione production with age in young male adrenals was due to an increasing *in vivo* effect of testicular testosterone on adrenal 3 β -hydroxysteroid dehydrogenase, Δ^{4-5} isomerase enzyme system activity. Thus our data support the concept that it is 'extra-adrenal' rather than 'intra-adrenal' factors that dominate in the differential modulation of androgens and cortisol production. However, the identity of this 'extra-adrenal' factor has yet to be elucidated.

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