Adrenarche in the rat

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Abstract

Normal pubertal development in humans involves two distinct processes: maturation of adrenal androgen secretion (adrenarche) and activation of the hypothalamic–pituitary–gonadal axis (gonadarche). One factor thought to contribute to the adrenarche in man is increased adrenal 17-hydroxylase (CYP17) activity. In the rat, there is evidence for adrenal involvement in the initiation of puberty, but the adrenal glands of this species are generally thought to express CYP17 only very poorly at best. To further examine the nature of postnatal adrenal development in rat, plasma samples and adrenal tissues were taken from animals aged 2–90 days, circulating adrenal

steroids assayed, and adrenal zones assessed quantitatively. A relative increase in zona reticularis, and peaks of circulating cortisol, androstenedione, and 17-OH-progesterone were observed around postnatal days 16–20, clearly before the development of the gonads, which begins at 30–35 days. Quantitative reverse transcriptase PCR confirmed a peak in mRNA coding for CYP17 in adrenal tissue from rats of similar age. The results suggest that the rat adrenal has the capacity to secrete steroids arising from 17-hydroxylation, and that this may contribute to a process similar to human adrenarche.

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Introduction

In addition to glucocorticoids and aldosterone, the human adrenal gland secretes other steroids, which are either true androgens or serve as precursors for androgen formation in peripheral tissues. Preceding puberty in man, the adrenal gland secretes increasing amounts of androgens, particularly dehydroepiandrosterone and its sulfate (Rosenfield & Eberlein 1969, Hopper & Yen 1975, Lee & Migeon 1975, Sizonenko & Paunier 1975, de Peretti & Forest 1976, Ducharme *et al.* 1976, Korth-Schutz *et al.* 1976, Reiter *et al.* 1977, Genazzani *et al.* 1978, Grumbach *et al.* 1978, Parker *et al.* 1978, Parker 1991, Parker & Odell 1980, Pintor *et al.* 1980, Rich *et al.* 1981, Smail *et al.* 1982, Perez-Fernandez *et al.* 1987). At the same time, a new cortical zone develops, the zona reticularis, which is often held to be responsible for the secretion of such C₁₉ steroids. The process is known as adrenarche (Albright *et al.* 1942, Dhom 1973, Parker & Odell 1980, Parker 1991) and has been described only in humans and higher primates (Cutler *et al.* 1978, Winter *et al.* 1980, Smail *et al.* 1982). The hormonal control of adrenal androgen secretion is poorly understood and the mechanisms responsible for adrenarche have been the subject of considerable investigation.

The participation of the adrenal gland in the maturation of the hypothalamic–pituitary–gonadal axis (with the adrenal androgens contributing to the activation of this axis) has been suggested, particularly because of the clear temporal sequence

between adrenarche and gonadarche (Boyar *et al.* 1973, Gorsky & Lawton 1973, Forest *et al.* 1975, Sizonenko 1978*a,b*, Cutler & Loriaux 1980, Ojeda *et al.* 1980, Smail *et al.* 1982, Katz *et al.* 1985, Parker 1991). This influence could be the result of one or a combination of the following mechanisms:

- direct action of the adrenal steroid hormones on the gonads;
- an action at the central nervous system (CNS), either facilitating the development of neuronal interconnections or inducing the secretion of gonadotropins or still, altering its pulsatile pattern;
- a permissive action (either at the gonads or at the CNS).

Alternatively, adrenarche may simply represent a progressive maturation in the activities of steroidogenic enzymes (such as 17,20 lyase) resulting from the increase in adrenal size, with no specific physiological significance.

In the rat, CYP17 has been generally considered to be absent from the adrenal cortex (Van Weerden *et al.* 1992, Pelletier *et al.* 2001) thus explaining why corticosterone, instead of cortisol, is the main glucocorticoid produced by the adrenal gland in this species. Nevertheless, several authors have reported the production of steroids arising from 17-hydroxylation (Bardin & Peterson 1967, Askari 1970, Kniewald *et al.* 1971, Milewich & Axelrod 1972, Vinson *et al.* 1976, 1978*a,b*, Bell *et al.* 1979), while other authors' findings are equivocal (Belanger *et al.* 1990, Leal & Moreira 1997).

However, the adrenals certainly influence the timing of puberty in the rat, and Corey & Britton (1931) first showed that the implantation of adrenal extracts was able to initiate early puberty. Moon (1937) then reported that vaginal opening did not occur following adrenalectomy, but this could be reversed by the administration of adrenocorticotropin (ACTH). Later, Gorsky & Lawton (1973) found that puberty is significantly delayed by adrenalectomy at 18 or 25 days postnatally, but not at 35 days. They also autotransplanted adrenal tissue into different groups of adrenalectomized rats and found that the pubertal delay was corrected when autotransplantation was performed at 18 days, but not at later times. They postulated that an adrenal factor was instrumental in the development of puberty, and since the adrenal autotransplants do not have a medulla, this factor must be of cortical origin.

Studies have also shown that male rat orchidectomy induces an increase in mitotic activity in the rat adrenal zona reticularis (Andò *et al.* 1989) and it also frequently causes the development of adrenal cortex tumors (Woolly *et al.* 1939, Houssay *et al.* 1953, Woolly 1953). Bell *et al.* (1978, 1979) demonstrated adrenal androstenedione production *in vitro*, although steroid secretion by zona reticularis cells was always inefficient when compared with the zona fasciculata. ACTH stimulation increases the output of steroids by both the two inner zones of the adrenal cortex. Supporting data were produced by Andò *et al.* (1988), who showed that castration in adult male rats induced the adrenal secretion of androstenedione and this tended to maintain the circulating levels of androgens. Finally, Rilianawati *et al.* (1998, 2000) using a transgenic mouse model for the study of gonadal tumorigenesis (inhibin α -subunit promoter/SV-40 T antigen) noted that gonadectomy induced the development of adrenal tumors and this phenomenon was gonadotropin-dependent. These facts implied the ectopic expression of luteinizing hormone (LH) receptors (together with increased levels of the ligand) in adrenal cortex tumorigenesis in these gonadectomized rodents, perhaps with the implication that the adrenal cortex can be stimulated to produce androgens.

The present studies were designed to examine the nature of adrenarche in the rat and test the possibility that changes in steroid output, including the secretion of androgens, might be involved.

Materials and Methods

Male and female Wistar rats were used. The animals were sacrificed at 2-day intervals between birth and postnatal day 20 and at 5-day intervals from day 20 until adulthood. The rats were kept under normal laboratory conditions with lights on from 0700 to 1900 h and a dark period from 1900 to 0700 h. Room temperature was kept constant at 20 °C. The animals were allowed free access to food and water. After weaning, which took place at around postnatal days 17–18, they were placed in cages according to the date of birth and

sex, with not more than three or four rats per cage. They were handled gently by the same operator to minimize stress.

The rats were killed by decapitation. This always took place between 0900 and 1100 h in order to avoid the effects of circadian variation. After death, truncal blood was collected, with and without EDTA, and after centrifugation, serum and plasma aliquots were kept at –20 °C (or –70 °C, if the interval before assay was expected to be long) for the hormone assays. The adrenal glands were also rapidly dissected, weighed, and fixed. Both Bouin fluid and buffered formalin were used. After dehydration, the glands were embedded in paraffin and sections of 5 μ m were cut and stained with hematoxylin and eosin for light microscopy. Only equatorial sections were used (i.e. those with the largest diameter) and at least three sections per rat were examined. The cortex and the zones were drawn using a *camera lucida* and their areas determined with an image analyzer (MOP Videoplan, Kontron Elektronik, Munich, Germany). The testes of the male rats were also dissected and weighed in order to confirm puberty. A minimum of ten animals was used at each time point.

Hormone assay

Corticosterone was assayed by HPLC. All other hormones were determined by RIA using commercial RIA kits (cortisol, Amersham; androstenedione, Incstar, Stillwater, MN, USA; 17-OH-progesterone, and testosterone, DPC, Los Angeles, CA, USA). The detection limits, cross-reactivities, and coefficients of variation (CV) are presented in Table 1.

Real-time reverse transcription and PCR (RT-PCR) and RT-PCR

Adrenal RNA from adult male Wistar rats (60 days, 200–300 g), obtained from the colony of the Gulbenkian Institute of Science, Oeiras, Portugal, as well as developing male and female rats of the same species resulting from breeding in the Faculty of Medicine of Porto, Portugal, was prepared using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and quantified spectrophotometrically by measuring absorbance at 260 and 280 nm. The total RNA purity (A_{260}/A_{280}) was between 1.6 and 1.9. The quality of RNA was confirmed by ethidium bromide staining after 1% agarose gel fractionation. The extracted RNA was stored at –70 °C until required.

Primers

For amplification of CYP17 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), primers were chosen from the previously published sequences (Horikoshi & Sakakibara 2000, Thompson *et al.* 2005). The sequences of the sense and antisense primers for CYP17 were 5'-TGGCT-TTCCTGGTGCACAATC-3' and 5'-TGAAAGTTGGT-GTTCGGCTGAAG-3' respectively, corresponding to bases 973–993 and 1062–1040. For GAPDH, sense and antisense

Table 1 Characterisation of radioimmunoassays

	Detection limit	Cross reactivities	Intra-assay CV (%)	Inter-assay CV (%)
Androstenedione	0.1 ng/ml	Corticosterone <0.01%	6.4	11.1
Cortisol	0.1 µg/dl	Cortisol 0.02%	5.7	8.9
		Cortisone 0.27%		
		Corticosterone 0.34%		
		11-deoxycortisol 3.17%		
17-OH-progesterone	0.07 ng/ml	17-OH-progesterone 1.91%	4.0	4.8
		Androstenedione 0.021%		
		Testosterone 0.008%		
		Cortisol 0.01%		
		Progesterone 3.5%		
Testosterone	0.04 ng/ml	Corticosterone 0.000 %	5.5	10.4
		Androstenedione 0.5%		
		Corticosterone 0.002 %		
		Cortisol 0.005%		

CV, coefficient of variation.

primers were 5'-CCC TCA AGATTG TCA GCA ATG C-3' and 5'-GTC CTC AGT GTA GCC CAG GAT-3' corresponding to bases 422–443 and 831–811 respectively.

Quantitative RT-PCR (QRT-PCR)

QRT-PCR was conducted, as previously described (Xiao *et al.* 2004), with minor modifications. Using Brilliant SYBR Green QRT-PCR Master Mix Kit, one-step, based on real-time detection of accumulated fluorescence (Mx300P; Stratagene, Amsterdam, The Netherlands), QRT-PCR was performed according to the manufacturer's protocol. Total cellular RNA, 200 ng, was used per reaction. QRT-PCR was carried out using the following time courses: 50 °C for 30 min (first-strand) cDNA synthesis, 95 °C for 10 min, and 45 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min for amplification. The amplified products were subjected to a stepwise increase in temperature from 55 to 95 °C and dissociation curves were constructed. The relative amount of each mRNA was normalized to the housekeeping gene (*GAPDH*) mRNA. Each sample was tested in triplicate.

Conventional RT-PCR

Reverse transcription was carried out at 23 °C for 20 min and at 42 °C for 60 min using 5 µg RNA, 1 µl reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase, M-MLV RT, 200 U/µl; Gibco) and 1 µl random hexamers (3 µg/µl; Gibco) in a 20 µl reaction mixture. Single-stranded cDNA (1 µl) in the 20 µl reaction mixture was amplified with 25 pmol each of sense and antisense primers, and 0.5 µl Taq DNA polymerase (5 U/µl) added to 50 µl PCR buffer – 20 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l each of dATP, dGTP, dCTP, and dTTP. The reactions were performed for 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C for 40 cycles with a final extension of 5 min at 72 °C. RT-PCR products were electrophoresed in 10 µl aliquots on

2.5% agarose gel. RT-PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced by Lark Technologies, Inc. (Essex, UK) and Qiagen GmbH (Hilden, Germany).

Statistical analysis

The significance of the differences found in the results was determined by ANOVA and the correlation between every parameter and postnatal age was studied by linear regression and significance determined by Student's *t*-test.

Results

The weights of the animals showed an almost linear growth until postnatal age of 70–75 days, in both males and females (correlation coefficients >0.95), and the same was true for adrenal weight. The area of the cortex (Fig. 1) and the areas of zonae fasciculata and reticularis (Figs 2 and 3) also increased from birth to adulthood with an attenuation of this growth after 60 days.

Figure 1 Development of the adrenal cortex in male and female rats during the postnatal period (mean ± S.E.M., *n* ≥ 10 throughout). **P* < 0.05.

Figure 2 Development of the zona fasciculata in male and female rats during the postnatal period (mean $\pm$ S.E.M., $n \geq 10$ throughout). * $P < 0.05$.

Figure 3 Development of the zona reticularis in male and female rats during the postnatal period (mean $\pm$ S.E.M., $n \geq 10$ throughout).

The adrenal weight as well as the area of the cortex (Fig. 1) and zona fasciculata (Fig. 2), but not the area of the zona reticularis (Fig. 3), exhibited sexual divergence after puberty, being larger in female rats than in males.

Although cells that were apparently zona reticularis could be identified earlier (and data indicating this is included in the figures), the zona reticularis became established as a clearly identifiable zone only after postnatal day 10. The ratio of the

zona reticularis area to the cortical area (zona reticularis/cortex) increased with time implying that the proportion of the cortical area occupied by the zona reticularis increased from birth and more specifically from postnatal day 10 to adulthood (Fig. 4).

Corticosterone, which is the major glucocorticoid produced by the rat adrenal cortex, increased linearly from birth to adulthood (Fig. 5). After a brief period in which levels were close to the detection limits (coinciding with the stress hypo-responsive period), corticosterone levels augmented progressively until 40 days of age. From then on, values stabilized in the males, while in females corticosterone levels continued to increase, to become stable only around 60 days, contributing to the significant differences between adult males and females.

In contrast, cortisol, 17-OH-progesterone and androstenedione increased both in male and female rats, without sex differences, between postnatal days 16 and 20 (Figs 6–8). These secretory peaks were clearly identifiable at a period which preceded puberty, as was confirmed by plotting testicular weight or the testosterone levels against postnatal age both of which confirmed that puberty takes place later, from 30/35 to 55/60 days after birth (Figs 9 and 10).

After 25/30 days of age, the cortisol and androstenedione peaks returned to very low levels, close to the detection limits of the RIA kits that were employed (Figs 6 and 7). In contrast, circulating 17-OH-progesterone again became elevated to give a later peak only in the female, possibly reflecting the development of the ovary (Fig. 8).

QRT-PCR

PCR products derived from developing rat adrenals are illustrated in Fig. 11. They showed the presence of components of an appropriate size for CYP17 and identical with those obtained from ovary and testis. The sequence data were obtained from nucleotides 973–1062 (NM 012753.1, Genbank) and sequences were identical to that expected for

Figure 4 Development of the adrenal cortex – area occupied by each zone. ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis.

Figure 5 Plasma corticosterone levels during the postnatal period (mean $\pm$ S.E.M., $n \geq 4$ throughout). * $P < 0.05$.

CYP17. This product was present in developing glands, but hybridization products with a similar melting point in QRT-PCR could not be quantified after 25–60 days of age (CYP17/GAPDH mRNA ratio < 0.0001).

Discussion

We first observed that morphological changes occur in the rat adrenal during the prepubertal period of development. One of these is the increase in adrenal size that at least partly results from the appearance of a new zone, the zona reticularis, which is seen as a clearly identifiable zone only after postnatal day 10, but afterwards it increases in size faster than the other zones (Figs 1–4). Although a specific function of this zone has never been ascertained in the rat, there is a striking resemblance with the development of zona reticularis in humans (Dhom 1973).

We also observed differences in the pattern of secretion of steroid hormones in this period. Corticosterone, the major steroid secreted by the adult rat adrenal, shows maturation of its secretory pattern in the peripubertal period, roughly 45–60 days, during which the characteristic sex dimorphism becomes apparent (Fig. 5). However, other steroids, not normally associated with the adult rat adrenal, were present in the circulating plasma during a period that clearly precedes gonadal maturation. These included androstenedione,

Figure 6 Plasma cortisol levels in male and female rats during the postnatal period (mean $\pm$ S.E.M., $n \geq 6$ throughout). Cortisol was almost undetectable until 14/16 days and again after 35, with a peak at day 20.

Figure 7 Plasma androstenedione levels in male and female rats during the postnatal period (mean $\pm$ S.E.M., $n \geq 7$ throughout). Androstenedione levels rise until 18–20 days, becoming very low after 35 days, until adulthood.

cortisol, and 17-OH-progesterone (Figs 6–8). All these products depend on CYP17 activity, an enzyme that is generally thought not to exist in the rat adrenal cortex (Fraser 1992, Van Weerden *et al.* 1992, Katagiri *et al.* 1998), for their synthesis, except perhaps in the fetus (Keeney *et al.* 1995). The secretory peaks of cortisol and androstenedione occurred between 16 and 20 days postnatally. The development of circulating testosterone occurred later, reaching its peak at 60 days (Fig. 9), which parallels changes in testis weight (Fig. 10). If this is considered to be an index of the maturation of the testis in males, it is unlikely that testicular CYP17 contributes to the earlier peaks in cortisol and androstenedione (Figs 5 and 6). Indeed, the secretory pattern of 17-OH-progesterone is particularly revealing; it shows two peaks, an early peak at about 20 days, coincident with those of cortisol and androstenedione, and the second in females only at 60+ days, resembling the rise in testosterone in the male, and perhaps similarly associated with gonadal development.

Figure 8 Plasma 17-OH-progesterone levels in male and female rats during the postnatal period (mean $\pm$ S.E.M., $n \geq 7$ throughout). A secretory peak around 20 days is observed. After 35 days, a second peak occurred in females, perhaps through ovarian secretion. * $P < 0.05$.

Figure 9 Plasma testosterone levels in male rats during the postnatal period (mean $\pm$ S.E.M., $n \geq 11$ throughout). The first point at which clearly elevated testosterone levels were observed was at day 35.

It seems likely that the early manifestation of CYP17 activity is attributable to the adrenal. The additional finding of transient transcription of CYP17 in the developing rat adrenal (Fig. 11) is consistent with the circulating steroid data and suggests that at this time, the adrenal may be a significant source of products arising from 17-hydroxylation. A burst of 17-OH-progesterone secretion has already been reported to take place between 10 and 20 days (Tsai & Johnson 1981) although in that study only females were studied.

The cortisol secretory peak deserves special consideration, since it is a phenomenon that does not occur in the human, where cortisol secretion is stable during the prepubertal and pubertal periods (Pintor *et al.* 1980). Although cortisol is not a functional glucocorticoid in the adult rodent (Fraser 1992, Katagiri *et al.* 1998), it has been shown to be the major glucocorticoid in neonatal rabbits and guinea pigs (Hardy *et al.* 1972, Malinowska *et al.* 1972).

Despite the reported absence of 17-hydroxylase in the rat adrenal, a number of previous studies have shown that steroid products arising from 17-hydroxylation can be formed from rat adrenal tissue *in vitro*, including cortisol, androstenedione, and testosterone, among others, albeit at a very low level (Askari 1970, Milewich & Axelrod 1972, Vinson *et al.* 1976, 1978a,b, Bell *et al.* 1979), while *in vivo*, adrenal production of androgen seems to be evident in the absence of the testis (Bardin & Peterson 1967, Kniewald *et al.* 1971).

Figure 10 Changes in testicular weight during the postnatal period (mean $\pm$ S.E.M., $n \geq 7$ throughout).

Figure 11 PCR of mRNA obtained from developing rat adrenals. Both female and male glands at 18 days (F18 and M18) gave PCR products of a size (90 base pairs, bp, cf. M, marker) expected for CYP17 and identical to those obtained with mRNA from ovary and testis. Quantitative RT-PCR (lower figure) showed that these products were present in the developmental period, but could not be quantified (Cyp17/GAPDH mRNA ratio < 0.0001) after 25–60 days of age (mean $\pm$ S.E.M., $n = 4$).

However, it remains possible that circulating cortisol or androgens could arise from the interaction between different organs and that the adrenal could merely provide precursors for CYP17 action at other sites, and an adrenal–testis interaction has been proposed (Feek *et al.* 1985, 1989). The testis is not the only possible site for conversion of adrenal products and in this connection it is important to note that the presence of 17-hydroxylase has also been demonstrated in rat hepatic cells, again between postnatal days 14 and 21 (Vianello *et al.* 1997, Katagiri *et al.* 1998). The possibility of such ‘cooperation’ between the liver and other tissues has been suggested in other situations, for example, in humans where the liver collaborates with the fetal adrenal and the placenta to produce oestriol (Bolander 1989) and with the skin and the kidney for the production of $1\alpha,25$ -dihydroxycholecalciferol (Bolander 1989).

Taken together, the morphological changes, the hormone assay data, and the presence of mRNA coding for CYP17 more strongly suggest that a true adrenarche is taking place in these rodents. The steroids produced at such a specific time of the rat prepubertal period may have a role in prepubertal development. In the human, this idea has been the subject of some dispute since precocious adrenarche may not always be followed by similarly precocious puberty (Sizonenko & Paunier 1975, Lee & Gareis 1976), and precocious puberty

may occur without prior adrenarche. (Sizonenko & Paunier 1975, Sklar *et al.* 1980, Counts *et al.* 1987). In the first case, it may be that steroids produced in the adrenal only produce their effects on prepared or primed gonads or hypothalamus; the second case suggests an independent pathology. In the normal situation, one possibility would be that androgens originating in the liver and adrenal gland are substrates for CYP18-mediated oestrogen synthesis by Sertoli or granulosa cells and hence help to support the proliferation of those cells (Vianello *et al.* 1997) in a period at which the gonads do not produce enough androgen substrate on their own.

However, cortisol cannot be converted into oestrogens. Thus, an alternative interpretation might be that this is a purely adrenal event, perhaps in response to a significant stress that might occur in this period. Curiously, it is precisely during this period that separation from the mother takes place. The specific functions of cortisol that are not shared by corticosterone are not clear. What is clear is that this is a real phenomenon, and its significance needs to be addressed.

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