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Alternative pathway androgen biosynthesis and human fetal female virilization

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Androgen biosynthesis in the human fetus proceeds through the adrenal sex steroid precursor dehydroepiandrosterone, which is converted to testosterone in the gonads, followed by further activation to 5α-dihydrotestosterone in genital skin, thereby facilitating male external genital differentiation. Congenital adrenal hyperplasia due to P450 oxidoreductase deficiency results in disrupted dehydroepiandrosterone biosynthesis, explaining undervirilization in affected boys. However, many affected girls are born virilized, despite low circulating androgens. We hypothesized that this is due to a prenatally active, alternative androgen biosynthesis pathway from 17α-hydroxyprogesterone to 5α-dihydrotestosterone, which bypasses dehydroepiandrosterone and testosterone, with increased activity in congenital adrenal hyperplasia variants associated with 17α-hydroxyprogesterone accumulation. Here we employ explant cultures of human fetal organs (adrenals, gonads, genital skin) from the major period of sexual differentiation and show that alternative pathway androgen biosynthesis is active in the fetus, as assessed by liquid chromatography–tandem mass spectrometry. We found androgen receptor expression in male and female genital skin using immunohistochemistry and demonstrated that both 5α-dihydrotestosterone and adrenal explant culture supernatant induce nuclear translocation of the androgen receptor in female genital skin primary cultures. Analyzing urinary sex steroid excretion by gas chromatography–mass spectrometry, we show that neonates with P450 oxidoreductase deficiency produce androgens through the alternative androgen pathway during the first weeks of life. We provide quantitative in vitro evidence that the corresponding P450 oxidoreductase mutations predominantly support alternative pathway androgen biosynthesis. These results indicate a key role of alternative pathway androgen biosynthesis in the prenatal virilization of girls affected by congenital adrenal hyperplasia due to P450 oxidoreductase deficiency.

In humans, the regulation of sexual differentiation is intricately linked to early development of the adrenal cortex (4, 8). Disorders affecting adrenal steroidogenesis commonly affect sexual differentiation, as exemplified by the multiple variants of congenital adrenal hyperplasia (CAH), which result either in inappropriate or disrupted androgen biosynthesis. This consequently causes disorders of sex development (DSDs), which can manifest with external genital virilization in newborn girls (46,XX DSD) or undermasculinization of external genitalia in male neonates (46,XY DSD) (9). The most common variant of CAH, 21-hydroxylase (CYP21A2) deficiency, manifests with 46,XX DSD, while 17α-hydroxylase/17,20-lyase (CYP17A1) deficiency results in 46,XY DSD.

The congenital adrenal hyperplasia variant cytochrome P450 oxidoreductase (POR) deficiency can manifest with both 46,XY androgen biosynthesis | congenital adrenal hyperplasia | alternative androgen pathway | 5α-dihydrotestosterone | human sexual differentiation

Significance

In the classic androgen biosynthesis pathway, testosterone is converted to 5α-dihydrotestosterone, a step crucially required for normal male genital virilization. Congenital adrenal hyperplasia (CAH) due to P450 oxidoreductase deficiency (PORD) is an inborn disorder that disrupts classic androgen biosynthesis. However, some affected girls present with severe genital virilization at birth. We hypothesized that this is explained by a prenatally active, alternative biosynthesis pathway to 5α-dihydrotestosterone. We show that adrenals and genital skin cooperate to produce androgens via the alternative pathway during the major period of human sexual differentiation and that neonates with PORD still produce alternative pathway androgens during the first weeks of life. This indicates that alternative pathway androgen biosynthesis drives prenatal virilization in CAH due to PORD.

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DSD and 46,XX DSD (10–12). POR plays a pivotal role as the obligatory electron donor to all microsomal cytochrome P450 enzymes, including CYP21A2 and CYP17A1, the latter catalyzing the biosynthesis of dehydroepiandrosterone (DHEA), the major precursor for testosterone biosynthesis. Consequently, POR deficiency (PORD) results in low circulating androgen concentrations, which readily account for 46,XY DSD, but fails to account for the severe virilization of external genitalia regularly observed in affected 46,XX neonates.

An explanation for this striking and seemingly contradictory genital phenotype in PORD has been lacking. We hypothesized that this apparent paradox could be explained by the existence of an alternative pathway to androgen production that generates DHT from 17α-hydroxyprogesterone (17OHP) during human fetal sexual differentiation, thereby bypassing the classic androgen biosynthesis pathway via DHEA and testosterone, as previously proposed by us (11) and others (13, 14). Elements of this pathway have been characterized in the fetal gonad of the tammar wallaby pouch young (15–17) and fetal opossum urogenital tract (18, 19). 17OHP accumulates in PORD but also in the most common CAH variant, 21-hydroxylase deficiency, and thus could feed into the proposed alternative pathway, if present in the fetus. Indirect biochemical evidence has indicated that the proposed alternative pathway is active postnatally in individuals with CAH due to 21-hydroxylase deficiency (20, 21) and may explain maternal virilization observed in pregnancies affected by PORD (22, 23). However, direct delineation of the putative alternative pathway during human fetal development, and in particular during the major period of sexual differentiation, has been lacking.

Here, we present conclusive evidence for the presence and activity of the alternative pathway in the human fetus, producing potent androgens during the major period of sexual differentiation, and show that human fetal female external genitalia respond sensitively to androgens during the same period. In concert, these findings define an alternative pathway for androgen biosynthesis during the critical period of sexual differentiation in the human fetus that represents an important mechanism to explain the prenatal virilization of female infants affected by CAH.

**Results and Discussion**

**Androgen Biosynthesis in the Human Fetus during Sexual Differentiation.** To ascertain the presence and activity of the hypothesized alternative androgen pathway, we performed incubations with male and female fetal adrenals, gonads, and genital skin, which were collected at 6 to 10 weeks’ gestation (wpc) as previously described (8). We separately added deuterated steroid substrates for each step of the alternative pathway and employed liquid chromatography–tandem mass spectrometry (LC-MS/MS) to identify the resulting products (SI Appendix, Figs. S1 and S2). Experiments were conducted at least in triplicate for each organ of either sex (SI Appendix, Figs. S2 and S3).

Explant incubations with female tissue showed that some 17OHP entered the classic androgen biosynthesis pathway, yielding androstenedione in adrenal, ovary, and genital skin. However, testosterone was detected in only 2 of 9 female genital skin incubations, and not at all in female adrenals and gonads (Fig. 1A). By contrast, all 3 female tissues (genital skin, ovaries, and adrenals) showed conversion of 17OHP and subsequent intermediates through all steps of the proposed alternative androgen pathway, with the end product, DHT, produced from 5α-androstenedione, with either 5α-androstanediol or 5α-androstanedione as intermediates (Fig. 1A).

Male fetal tissues readily converted 17OHP along the classic androgen pathway to testosterone and, in the alternative pathway, to 5α-17-hydroxy pregnanolone (5α-17HP). In male genital skin, conversion from 17OHP proceeded until the generation of DHT.
of the 2 isoforms, the first step is expected to require catalysis by steroid 5α-reductase type 1 (SRD5A1), since SRD5A2 does not convert 17OHHP efficiently (30). A study of fetal tissues from 12 to 20 wpc did not detect SRD5A1 in adrenals, gonads, or genital skin (31); a recent study also using fetal tissues from the second trimester of pregnancy (11 to 21 wpc) described detection of SRD5A1 in liver, placenta, testis, and genital tubercle but not in the adrenal, while female gonads were not studied (32). However, studying tissue from the major period of human sexual differentiation, we found mRNA expression of SRD5A1 in adrenals, gonads, and genital skin of both sexes (Fig. 2A). The second step, the conversion of 5α-pregnane-17α-ol-3,20-dione to 5α-17HP, requires 3α-hydroxysteroid dehydrogenase activity, in keeping with the observed expression of AKR1C1 and AKR1C3, both of which encode enzymes capable of catalyzing this reaction (Fig. 2A).

The next step in the alternative pathway requires CYP17A1 17,20-lyase activity; 5α-17HP is the preferred substrate for this reaction and efficiently converted to 5α-androsterone (33). We detected robust CYP17A1 expression in human fetal adrenals from the major period of sexual differentiation, consistent with previous reports (8, 34) and also in the gonads of both sexes (Fig. 2B). The subsequent reduction of 5α-androsterone to 5α-androstenediol requires 17β-hydroxysteroid dehydrogenase activity, which can be provided by AKR1C3 or AKR1C1, expressed in adrenals, gonads, and genital skin (Fig. 2A).

The final step of the proposed pathway involves the conversion of 5α-androstenediol to DHT, which requires 3β-epimerase (oxidative 3α-HSD) activity. Several enzymes have been considered to catalyze this reaction (i.e., AKR1C2, RDH5, DHRS9, HSD17B10, HSD17B6, and RDH16). However, only HSD17B6 and RDH16 are capable of efficient oxidation of 5α-androstenediol to DHT, as previously demonstrated by transactivation of the androgen receptor following cell-based overexpression (24, 35). We found expression of both HSD17B6 and RDH16 in fetal genital skin of both sexes (Fig. 2C).

**Fig. 2.** Steroidogenic enzyme expression in human fetal tissue from the major period of sexual differentiation and their proposed role(s) in alternative pathway synthesis. (A and B) mRNA expression (mean ± SEM) in human fetal tissues collected at 6 to 10 wpc as measured by qPCR in female adrenals (n = 1), gonads (n = 2), genital skin (n = 2), and corresponding male tissues (≥3 biological replicates for adrenals, gonads, and genital skin). (C) Fetal genital skin mRNA expression of all enzymes potentially capable of converting 5α-androstenediol to 5α-dihydrotestosterone. Expression data were normalized to ribosomal 18S. (D) Schematic summary of the proposed distinct roles of the identified enzymes in the classic androgen pathway (dark blue) and the alternative androgen synthesis pathway (light blue), both resulting in the synthesis of potent 5α-dihydrotestosterone. Arrows indicate observed conversions in the fetal organ explant cultures; dotted arrows represent reactions only rarely observed.
In summary, we detected the transcripts encoding all enzymes required to catalyze the alternative androgen pathway (Fig. 2D). Taken together with the steroid conversion studies, these data comprehensively demonstrate that the normal adrenal, gonad, and genital skin are capable of androgen biosynthesis via both the classic and alternative pathways in both sexes. Our data point to an adrenogenital steroidogenic unit that can cooperate to produce DHT via the alternative pathway during the major period of human sexual differentiation.

**Androgen Receptor in Female Genitalia from the Start of Sexual Differentiation.** Having demonstrated the capacity for DHT production from steroidogenic precursors in female fetuses, we corroborated its ability to function by examining the presence of the androgen receptor (AR) in female external genitalia from the start of sexual differentiation. Previously, AR expression was documented in 4 female human fetuses from 9 to 18 wpc (36). In our study, we readily detected AR protein in stromal cells in the urethral folds of the external genitalia in both male and female fetuses at the onset of sexual differentiation (Fig. 3A–C). Its nuclear localization in fixed tissues in both sexes implied AR was ligand-bound. To explore this further, we studied the intracellular localization of AR by immunofluorescence in female external genital fibroblasts taken into primary culture from the same stage of development. In steroid-free media, the external genital cells demonstrated cytoplasmic AR localization. As expected, the addition of 1 nM DHT induced nuclear translocation of AR. Strikingly, the same translocation was observed when using medium conditioned overnight from the corresponding female adrenal gland (Fig. 3D). In combination, these data show AR from the start of sexual differentiation in both male and female external genitalia and demonstrate a functional adrenogenital steroidogenic unit capable of causing AR nuclear translocation.

**Androgen Biosynthesis in Neonates with Congenital Adrenal Hyperplasia Due to P450 Oxidoreductase Deficiency.** Having shown evidence of the alternative pathway during human sexual differentiation, we next investigated whether we could demonstrate equivalent activity in vivo. To address our hypothesis that excess alternative pathway androgen biosynthesis in fetal life explains female virilization (46,XX DSD) in CAH due to PORD (12, 37), PORD A287P demonstrated significantly higher activity than POR in the alternative androgen pathway than in the classic pathway. Our data point to an adrenogenital steroidogenic unit that can cooperate to produce DHT via the alternative pathway during the major period of human sexual differentiation.

![Fig. 3. Androgen receptor is present in both male and female genitalia from the onset of sex differentiation.](image)

(A) Morphology of male and female external genitalia at the onset of sex differentiation. (B) Immunofluorescence for AR in male (8) and female (C) genitalia at the start of human sexual development at 7 to 8 wpc counterstained with toluidine blue. Boxes (Left) are shown at higher magnification (Right). (D) Immunofluorescence for AR in female genital fibroblasts in the presence (+) or absence (−) of 10 nM DHT (Left) or medium conditioned by overnight incubation with an adrenal gland from the same female fetus (Right). Scale bars, 500 μm (A); 100 μm (low) and 20 μm (high magnification) (B and C); and 25 μm (D).
In conclusion, we have provided in vitro, ex vivo, and in vivo evidence for the existence and activity of an alternative pathway for the synthesis of the most potent androgen, DHT, during early human development. Our data demonstrate that, through cooperation of an adrenogenital steroidogenic unit, the alternative androgen pathway yields active androgen synthesis in the female fetus, with excess activity driving female virilization, 46,XX DSD, androgen pathway yields active androgen synthesis in the female fetus, with excess activity driving female virilization, 46,XX DSD, in CAH due to P450 oxidoreductase deficiency. Given that the alternative pathway substrate 17OHP also accumulates in 21-hydroxylase deficiency, it is conceivable that alternative pathway androgens contribute to prenatal virilization in this most common CAH variant.

Materials and Methods
Collection of Human Embryonic and Fetal Material. Ethical approval for these studies was granted by the North West Haydock Research Ethics Committee of the UK Health Research Authority (approval no. 18/NW/0096). The collection and staging of human embryonic and fetal material were carried out with informed consent, as described previously (8, 39), using Carnegie classification and fetal foot length to provide a direct assessment of developmental age as days or weeks post conception (dpc or wpc), respectively, and fetal foot length to provide a direct assessment of developmental age as days or weeks post conception (dpc or wpc), respectively, and male fetal material was identified by SRY expression, as previously described (1). We analyzed organs and tissue from 30 fetuses: 25 male and 5 female; median age 55 dpc (range 44 to 84 dpc).

RNA Extraction, Reverse Transcription, and Quantitative PCR. Total RNA was extracted from whole organs using the TRI Reagent system (Sigma-Aldrich). RNA integrity and concentrations were assessed using a NanoDrop spectrophotometer. Reverse transcription was carried out employing a standard protocol. mRNA expression levels were quantified using an ABI 7500 sequence detection system (PerkinElmer Applied Biosystems), employing the Applied Biosystems "assay on demand" probe and primers for specific amplification of SRDSA1, SRDSA2, CYP17A1, AKR1C1, AKR1C2, AKR1C3, AKR1C4, HADH2/HSD17B10, HSD17B6, DHR59, and RDH16 (for further details, see SI Appendix).

Tissue Explant Culture and Steroid Identification by Tandem Mass Spectrometry. Whole-organ tissue explants (fetal adrenals, gonads, and genital skin) were cultured in DMEM/F12 (PAA Laboratories) supplemented with 2% Ultroser SF (i.e., steroid-free; BioSepra) and 1% ITS (BD) at 37 °C in humidified 5% CO2 and 95% air for 64 h. Genital skin was cultured as monolayers and used for experiments at passage 4.

Identification of steroid products from the explant cultures was achieved using LC-MS/MS. Steroids were positively identified by comparison of retention time and MS/MS mass transitions with authentic steroid standards (SI Appendix, Table S1). Two mass transitions were used to positively identify each steroid, referred to as qualifier and qualifier ions, respectively; the resolution of a series of authentic steroid standards is shown in SI Appendix, Fig. S2, alongside further method details.

For steroid conversion assays, tissue explants were incubated with precursor steroids purchased from Steraloids and Sigma-Aldrich. For explant cultures assessing the conversion of 17-hydroxyprogesterone, 5α-androstanediol, we used deuterated steroids (for details, see SI Appendix, Table S2). Representative results of steroid detection following explant culture incubations are shown in SI Appendix, Figs. 53 and 54 for female and male tissues.

Immunohistochemistry. Immunohistochemistry, immunoblotting, and immunofluorescence were carried out as reported previously (40), using monoclonal mouse anti-AR (1:100; LabVision).

Urine Steroid Metabolite Excretion Analysis. Urine samples were collected longitudinally from birth until 90 d of life in 3 neonates affected with PORD (2 homozygous for POR A287P, and the other 1 compound-heterozygous for POR A287P/G188_V191dup) and compared with those collected from 9 healthy controls. These were matched for sex, age, and gestational age at birth and collected during the same time window. The parents of PORD patients and healthy controls provided written informed consent prior to urine collection. The study protocol was reviewed and approved by the Research Ethics Committee of the University College London Institute of Child Health/Great Ormond Street Hospital NHS Trust (REC reference 05/Q0508/24).

Urine steroid hormone profiles were determined by gas chromatography–mass spectrometry analysis as described previously (41). The final analytical samples are the methyloxime-trimethylsilyl derivatives of steroids.

![Fig. 4. Urinary steroid excretion in 3 46,XY neonates with POR deficiency (closed symbols) in comparison with 9 sex- and age-matched healthy controls (open symbols). POR-deficient neonates harbored the A287P mutation, which in the homozygous state is associated with normal male genitalia in boys and genital virilization (46,XX DSD). We included data from 3 46,XY neonates with PORD; 2 harbored homozygous POR mutations (A287P/A287P), and 1 harbored compound-heterozygous mutations (A287P/G188_V191dup). Longitudinal urine collections were carried out during the first 3 mo of life and analyzed by gas chromatography–mass spectrometry. Depicted are the urinary excretion of (A) the 17OHP metabolite 17-hydroxyprogrenalone (17HP) and the 2 alternative pathway intermediates (B) 5α-17-hydroxyprogrenalone (5α-17HP) and (C) 5α-androsterone (An), in comparison with (D) etiocholanolone (Et), which is only generated via the classic androgen pathway. All steroids are shown relative to tetrahydrocortisone (THE), an abundant adrenal-derived steroid metabolite, as the denominator.](https://www.pnas.org/cgi/doi/10.1073/pnas.1906623116)
enzymatically released from sulfate and glucuronide conjugation. Analytes quantified by selected ion monitoring were normalized to tetrahydrocortisone, the most abundant steroid metabolite consistently excreted throughout life, with no significant difference in urinary tetrahydrocortisone concentrations identified between PORD patients ($n = 3$; 13 urine samples; median 285 μg/L, range 56 to 1,256 μg/L) and healthy controls ($n = 9$; 48 urine samples; median 306 μg/L, range 59 to 1,663 μg/L).

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