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21-Hydroxylase deficiency in the neonate – trends in steroid anabolism and catabolism during the first weeks of life

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ABSTRACT

Deficiency of 21-hydroxylase provides an *in vivo* model of intrauterine induction of enzymes participating in steroid anabolism and catabolism. Quantitative data for 93 steroid metabolites in urine from 111 patients and 7 controls (25 samples) were compared over the first six weeks of life. Net flux through the key anabolic enzymes was examined by comparison of the totals of steroids derived from the intermediates prior to and following each enzymatic step. Metabolic relationships were established on structural grounds and by Pearson correlation. The relative importance of each catabolic route was evaluated after summing metabolites classified according to their structure as fetal, neonatal, and classical (adult) type.

Hierarchical cluster analysis identified the structure at C3–C5 as a key distinguishing feature of the major catabolic streams and demonstrated a split point in metabolic pattern in patients at 7 days. Changes with time in steroid metabolism, larger in patients than in controls, could be interpreted as reflecting increased cortisol demand *post partum*, the clinical onset of salt-wasting and a transition in catabolism from fetal to postnatal life. Faster involution of the *fetal zone* and pronounced enhancement of steroid production in *zona fasciculata* and *zona glomerulosa* were indicated in patients. Predominant at birth were 'planar' fetal-type 5α-reduced metabolites, adapted to placental excretion, which gave way to additionally hydroxylated neonatal-type metabolites, facilitating renal excretion. Classical metabolism made gains over the study period. Overproduction of steroids in utero in 21-hydroxylase deficiency would have induced fetal catabolic pathways dependent on 5α-reduction. A progressive increase of steroids likely to arise from 5α-reductase type 2 activity, again more distinct in disease, was observed.

We demonstrate that the key intermediates in the hypothetical 'backdoor' pathway of androgen synthesis are part of a broader catabolic network and should not be examined in isolation.

1. Introduction

Deficiency of 21-hydroxylase (CYP21A2), the commonest cause of congenital adrenal hyperplasia [1], can be considered an *in vivo* model of intrauterine induction of steroid metabolizing enzymes involved in both steroid anabolic and catabolic pathways.

The block along the anabolic pathway of cortisol synthesis results in the excessive production of cortisol precursors, especially 17-hydroxyprogesterone, and its by-products, i.e. 21-deoxycortisol and androstenedione, presenting both a catabolic and an excretory challenge to the developing fetus. This demand is superimposed on the normal physiological changes involved in fetal preparation for, and post-natal adaptation to, extra-uterine life which take place in the adrenals, liver, and kidneys. The result is the presence of a variety of steroids in urine from affected neonates that is unmatched in any other steroid metabolic disorder. We have presented their characterization in the preceding four parts of this comprehensive steroidomic project [2–5]. The present study utilizes quantification of these over the first six weeks of life in order to evaluate the activities of the two key anabolic enzymes involved in determining the fate of the universal steroid precursor pregnenolone. These are 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) and CYP17, which combines the independently regulated 17α-hydroxylase and 17-20-lyase activities [6]. The expression and biological activity of these enzymes in the three adrenal zones – the *definitive zone* of the fetal adrenal, an analog of *zona glomerulosa* of the adult gland, the *transient zone*, equivalent to *zona fasciculata*, and the *fetal zone*, equivalent to *zona reticularis* [7], determine the generation of the corresponding products, aldosterone, cortisol, and dehydroepiandrosterone (DHEA). The synthetic pathways do, however, partly overlap in two or in all three zones [6]. Levels of steroid

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metabolites in urine would reflect their combined effect and an evaluation of the individual enzyme steps cannot be achieved separately for each zone. Instead, we chose to examine net production of steroid metabolites derived from steroid precursors on either side of each enzyme step, comparing patients and controls during the first six weeks of life. These results were then interpreted in the context of known physiological and pathological processes and the potential involvement of the separate adrenal zones was thus inferred.

We have also paid particular attention to the net effect of 5α-reduction, for two reasons. First, this is potentially involved in the hypothesized anabolic 'backdoor' pathway of alternative production of dihydrotestosterone (DHT) from *allo*17-hydroxypregnanolone (17P(3α,5α)), often abbreviated 'Pdiol', via androsterone (A(3α,5α)), by-passing the classical pathway via androstenedione (AD) and testosterone (T). This was first considered significant in patients with cytochrome P450 oxido-reductase (POR) deficiency [8] and has more recently been claimed to be active in patients with 21-hydroxylase deficiency [9]. Second, the interest in 5α-reductases is driven by their involvement in the generation of a multitude of further oxygenated C_{19} as well as C_{21} 5α-reduced metabolites that we have previously described in urine from neonates with 21-hydroxylase deficiency [3,4]. These have not so far been taken into account in the evaluation of the metabolic pathways in this disorder.

1.1. Abbreviations and trivial names of steroids

F, cortisol (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione); **E**, cortisone (17α,21-dihydroxy-pregn-4-ene-3,11,20-trione); **EM**, E metabolites; **(*a*)THE**, (allo)tetrahydrocortisone (3α,17α,21-trihydroxy-5(α)β-pregnane-11,20-dione); **α(β)-cortolone** (3α,17α,20α(β),21-tetrahydroxy-5(α)β-pregnan-11-one); **20α(β)-dihydrocortisone** (17α,20α(β),21-trihydroxy-pregn-4-ene-3,11-dione); **11-deoxycortisol** (17α,21-dihydroxy-pregn-4-ene-3,20-dione); **21dF**, 21-deoxycortisol (11β,17α-dihydroxy-pregn-4-ene-3,20-dione); **21dE**, 21-deoxycortisone (17α-hydroxy-pregn-4-ene-3,11,20-trione); **21dEM**, 21dE metabolites; **(a)21dTHE**, (*allo*)21-deoxy-tertahydrocortisone (3α,17α-hydroxy-5(α)β-pregnane-11,20-dione); **11oxoP3**, 11oxo-pregnanetriol (3α,17α,20α-trihydroxy-5β-pregnan-11-one); **17Prog**, 17-hydroxyprogesterone (17α-hydroxy-pregn-4-ene-3,20-dione); **17P(3α,5α)**, *allo*17-hydroxypregnanolone (3α,17α-dihydroxy-5α-pregnane-20-dione); **17ProgM**, 17Prog metabolites; **5α(β)17ProgM**, 5α(β)-reduced metabolites of 17Prog; **6oxo17Prog**, 6oxo-17-hydroxyprogesterone (17α-hydroxy-pregn-4-ene-3,6,20-trione); **6oxo17ProgM**, 6oxo17Prog metabolites; **11oxoProg**, 11oxo-progesterone (pregn-4-ene-3,11,20-trione); **11oxoP2**, 11oxo-pregnanediol (3α,20α-dihydroxy-5β-pregnan-11-one); **Prog**, progesterone (pregn-4-ene-3,20-dione); **ΔP**, pregnenolone (3β-hydroxy-pregn-5-en-20-one); **ΔPM**, ΔP metabolites; **17ΔP**, 17-hydroxypregnenolone (3β,17α-dihydroxy-pregn-5-en-20-one); **17ΔPM**, 17ΔP metabolites; **7oxo17ΔP**, 7oxo-17-hydroxypregnenolone (3β,17α-dihydroxy-pregn-5-ene-7,20-dione); **7oxo17ΔPM**, 7oxo17ΔP metabolites; **DHEA**, dehydroepiandrosterone (3β-hydroxy-androst-5-en-17-one); **DHEAM**, DHEA metabolites; **AD**, androstenedione (androst-4-ene-3,17-dione); **ADM**, AD metabolites; **DHT**, dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); **androsterone** (3α-hydroxy-5α-androstan-17-one); **etiocholanolone** (3α-hydroxy-5β-androstan-17-one); **PLS-DA**, partial least squares-discriminant analysis; **3βHSD**, 3β-hydroxysteroid dehydrogenase, Δ4-5-isomerase.

2. Experimental

2.1. Materials

All materials were supplied as previously described [2,3].

2.2. Urine samples

Urine samples from 111 untreated neonates with 21-hydroxylase deficiency were sent by various centers to the host lab's referral service as a part of their clinical investigation and have been previously described [5]. For the investigation of the general metabolic trends they were placed in two categories based on postnatal age (rationale explained in Section 3.3) as follows: 0–7 days ('**Young**') – $n = 67$ (4 males) – mean 2.6 days (95% confidence interval 2.2–3.1 days); 8–46 days ('**Old**') – $n = 44$ (32 males) – 15.5 days (13.5–17.7 days). An examination of the evolution of 5α/5β ratios with postnatal age established a rise at the end of week four of life, which determined the subdivision of the 'Old' group with a split point 27 days for the investigation of the indices of 5α/5β reduction (Section 3.6). The sub-groups of the **'Old'** group were as follows: 8–27 days ('**Older**') – $n = 38$ (26 males) – mean 13.6 days (12.6–14.7); 28–46 days ('**Oldest**') – $n = 6$ (all male) – mean 34.7 days (33.1–36.3). Control samples ($n = 25$ for 7 subjects (4 males)) have also been previously described [5]. These were correspondingly grouped as follows: 0–4 days ('**Young**'), $n = 11$ (7 males); 8–29 days ('**Old**'), $n = 14$ (8 males) with further sub-divisions 8–10 days ('**Older**'), $n = 7$ (4 males) and 29 days ('**Oldest**'), $n = 7$ (4 males).

2.3. Steroid analysis and quantification

Steroid analysis, derivatization and GC–MS were carried out as previously described [2]. In brief, following solid phase extraction on C_{18} silica-containing cartridges (Sep-Pak, Waters, UK), steroid conjugates were subjected to hydrolysis with *Helix pomatia* juice (Biosepra, Cergy, France) in 0.5 M acetate buffer at pH 4.6 with the addition of sodium ascorbate (incubated for 72 h at 37 °C). The freed steroids were then subjected to a further solid phase extraction (on C_{18} cartridges) and derivatization, forming methyloxime-trimethylsilyl (MO-TMS) derivatives, which were analyzed in a gas-chromatograph fitted with a 100% dimethylpolysiloxane column and coupled to a single quadrupole mass-spectrometer. The quantification approach has also been described [4]. This was based on extracted ion chromatograms of an ion characteristic of each compound (Table 1). When a synthetic standard was not available, the calibration curve of an available standard with the nearest structure, or the nearest qualitative match of the spectrum, was used.

The quantitatively major and structurally important steroid metabolites ($n = 80$) considered in this presentation were linked in a metabolic chart based on their structure (Fig. 1). Full systematic names are listed in Table 1. Metabolite labels follow the format: **letter**, corresponding to the article in which the metabolite has been previously reported and **number**, corresponding to the number given to this metabolite in retention time order. The letter abbreviations of the articles are as follows: **A** – androstane(ene)s [4]; **B** – pregnane(ene)s with an oxo group on the A or B-ring [3]; **C** – 11oxo-pregnane(ene)s [5]; **D** – pregnane(ene)s containing only hydroxyls outside the D-ring [2]. In addition, 11oxoP2 (**C6**) and 12 cortisone metabolites were quantified. These comprised (a) THE – (allo)-tetrahydrocortisone **C9**(**C14**); α(β)-cortolone **C19**(**C23**); 6α-hydroxyTHE **C16**; 1β-hydroxyTHE **C21**; 6α-hydroxy-α(β)-cortolone **C29**(**C34**); 1β-hydroxy-β-cortolone **C36**; cortisone **C43**; 20α(β)-dihydrocortisone **C47**(**C49**).

Metabolites were grouped into the following three types, as shown in Fig. 1: **classical** or adult-type, which are those that are also present in adults, **neonatal-type**, which encompass the further