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BACKGROUND: The clinical differential diagnosis of classic 21-hydroxylase deficiency (C21OHD) and cytochrome P450 oxidoreductase deficiency (PORD) is sometimes difficult, since both deficiencies can have similar phenotypes and high blood concentrations of 17α-hydroxyprogesterone (17OHP). The objective of this study was to identify biochemical markers for the differential diagnosis of C21OHD, PORD, and transient hyper 17α-hydroxyprogesteronemia (TH17OHP) in Japanese newborns. We established a 2-step biochemical differential diagnosis of C21OHD and PORD.

METHODS: We recruited 29 infants with C21OHD, 9 with PORD, 67 with TH17OHP, and 1341 control infants. All were Japanese and between 0 and 180 days old; none received glucocorticoid treatment before urine sampling. We measured urinary pregnanetriolone (Ptl), the cortisol metabolites 5α- and 5β-tetrahydrocortisone (sum of these metabolites termed THEs), and metabolites of 3 steroids, namely dehydroepiandrosterone, androstenedione (AD4), and 11β-hydroxyandrostenedione (11OHD4) by GC-MS.

RESULTS: At a cutoff of 0.020, the ratio of Ptl to THEs differentiated C21OHD and PORD from TH17OHP and controls with no overlap. Among metabolites of DHEA, AD4, and 11OHD4, only 11β-hydroxyandrosterone (11HA), a metabolite of 11OHD4, showed no overlap between C21OHD and PORD at a cutoff of 0.35 mg/g creatinine.

CONCLUSIONS: A specific cutoff for the ratio of Ptl to THEs can differentiate C21OHD and PORD from TH17OHP and controls. Additionally, the use of a specific cutoff of 11HA can distinguish between C21OHD and PORD.

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Classic 21-hydroxylase deficiency (C21OHD)6 is the most common form of congenital adrenal hyperplasia, which is transmitted as an autosomal recessive trait. C21OHD is caused by mutations of CYP21A2 (cytochrome P450, family 21, subfamily A, polypeptide 2)7 encoding 21-hydroxylase, which catalyzes steroid hydroxylation at C21 (Fig. 1) (1). C21OHD shows adrenal insufficiency, disorders of sex development in 46,XX, and increased serum 17α-hydroxyprogesterone (17OHP). Cytochrome P450 oxidoreductase (POR) deficiency (PORD) is a recently established form of congenital adrenal hyperplasia that is also transmitted as an autosomal recessive trait. PORD is caused by mutations of POR encoding POR, which transfers electrons to microsomal P450 enzymes such as 17α-hydroxylase/17,20-lyase, 21-hydroxylase, and aromatase (Fig. 1) (2). PORD shows adrenal dysfunction,
disorders of sex development in 46,XX and 46,XY, skeletal dysplasia, maternal virilization during pregnancy, and increased serum 17OHP. In addition to increased concentrations of 17OHP, clinical manifestations of C21OHD and PORD can be similar, leading to difficulty in differential diagnosis (3, 4). We previously reported the biochemical differential diagnosis of C21OHD from transient hyper 17OHPnemia (TH17OHP) and controls in term and preterm neonates by measuring urinary pregnanetriolone (Ptl), which was a final metabolite of 21-deoxycortisol (21DOF) (5). Shackleton et al. (6) reported biochemical differential diagnosis of PORD from controls by a distinctive steroid excretion pattern, namely low urinary metabolites of cortisol and androgens and high metabolites of pregnenolone and progesterone. We reported that PORD had high urinary Ptl concentrations and that the ratio of 11β-hydroxyandrostosterone (11HA) to pregnanediol (PD) could differentiate PORD from C21OHD in 3 infants between the ages of 1 and 3 months (3). However, no cutoff for urinary steroid metabolites has been reported at any age for distinguishing between C21OHD and PORD. In our laboratory, the measurement of PD in newborns is sometimes problematic owing to unknown interferences, and we have not been able to calculate the ratio of 11HA to PD for the differential diagnosis of C21OHD and PORD.

The objective of this study was to identify biochemical markers for the differential diagnosis of C21OHD, PORD, and TH17OHP and to set the cutoff in Japanese infants <6 months old, the period during which most patients with C21OHD or PORD are diagnosed (7). We paid attention to 21-hydroxylase and 17,20-lyase activities since, theoretically, the former enzymatic activity is impaired in both C21OHD and PORD whereas only 21-hydroxylase is reduced in C21OHD.

Materials and Methods

All legal guardians gave written informed consent, and the study was approved by the institutional review board committee at Keio University Hospital. We recruited 29 infants with C21OHD, 9 with PORD, 67 with TH17OHP, and 1341 control infants from 2000 through 2009 at Keio University Hospital and 45 other hospitals throughout Japan (Table 1). All infants were
Japanese, with ages between 0–180 days. The diagnosis of C21OHD and PORD was confirmed by CYP21A2 and POR gene analysis, respectively (Table 2). The diagnosis of TH17OHP was made in the neonates fulfilling all the following criteria; (a) 17OHP concentration in the dried blood spot mass screening program in Japan (direct ELISA assay), (b) blood 17OHP concentration confirmed to be normal by repeated measure-

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<sup>a</sup> Del or conv, deletion or large gene conversion; E6 cluster, cluster of mutations (I236D, V237E, M239K) in exon 6; I2 splicing, intron 2 – 13 A/C>G; Δ8bp, 8-bp deletion in exon 3.
ments [ELISA assay (direct or extraction method) or RIA], and (c) consistently good overall general condition. Any subjects with abnormal physical findings were excluded. The control infants were without neurologic and endocrinologic abnormalities, and none of the subjects received antenatal or perinatal glucocorticoid before urine sampling. Table 1 shows characteristics of the study subjects.

Spot urine samples were randomly collected for the study and kept at −20 °C until analysis. We studied each infant for urinary steroid profile by GC-MS/selected ion monitoring (GC-MS-SIM), as reported (8) with minor modification. In brief, 0.05- to 0.2-mL urine samples were subjected enzymatic hydrolysis and organic solvent extraction and methyloxime-trimethylsilyl derivatized, and the derivative was subjected to GC-MS-SIM analysis. GC-MS-SIM analysis was performed on an HP5890II GC with an HP-Ultra1 fused silica column (25 m × 0.2 nm × 0.33 μm) coupled to an HP5971MS (Agilent Technologies). We quantified each steroid with stigmasterol as the internal standard. The turnaround time of the assay is 2 days.

We measured Ptl and the cortisol metabolites 5α-tetrahydrocortisone and 5β-tetrahydrocortisone (sum of these metabolites termed THEs) and calculated the ratio of Ptl to the cortisol metabolites (Ptl/THEs) (5, 9) to differentiate C21OHD and PORD from TH17OHP and controls. Ptl was considered to be equal to 0.001 mg/g creatinine for calculation in infants whose Ptl was under the detection limit (<0.001 mg/g creatinine). We measured metabolites of 3 steroids, namely dehydroepiandrosterone (DHEA), androstenedione (AD4), and 11β-hydroxyandrostenedione (11OHD4), and calculated the sum of DHEA metabolites (DHEA, androsterone, etiocholanolone, 16α-hydroxy-DHEA, 16β-hydroxy-DHEA, 16-oxo-androstenediol, and androstenediol), the sum of AD4 metabolites (androsterone and etiocholanolone) (8, 10), and the 11OHD4 metabolite (11HA). (11β-Hydroxyetiocholanolone could not be measured in newborns, as described (8.).) The above sum of DHEA metabolites have been reported to include approximately 70% of DHEA metabolites in newborns (11). The above sum of AD4 metabolites are defined as androgen metabolites because, whereas these 2 steroids are metabolites of AD4, they are also the metabolites of testosterone and dihydrotestosterone in boys. Androgen metabolites are analyzed separately by sex, since male androgen metabolites in this age group are increased from testicular-derived androstenediol and etiocholanolone.

We measured urinary creatinine by IATRO-LQ CRE (A)II (Mitsubishi Chemical Medience Co.) and expressed urinary steroid concentration relative to urinary creatinine (mg/g creatinine).

Statistical analysis of DHEA, androgen, and 11OHD4 metabolites was carried out by Mann–Whitney U-test between C21OHD and PORD. A P value of <0.05 was considered statistically significant.

Results

DIFFERENTIATION OF C21OHD AND PORD FROM TH17OHP AND CONTROLS

Fig. 2 shows the results of urinary Ptl and Ptl/THEs. Ptl [median (range) mg/g creatinine] was 10 (0.079–36) in C21OHD, 1.5 (0.42–2.6) in PORD, 0.006 (<0.001–0.086) in TH17OHP, and <0.001 (<0.001–0.064) in controls. Ptl/THEs was 2.5 (0.14–15) in C21OHD, 0.18 (0.051–0.23) in PORD, 0.0083 (0.00010–0.0011) in TH17OHP, and 0.00038 (0.000068–0.00083) in controls. Ptl differentiated C21OHD and PORD from TH17OHP and controls with 96.9% (95% CI 91.6%–97.4%) sensitivity and 100% (99.8%–100%) specificity with cutoff 0.1 mg/g creatinine. All patients with TH17OHP and controls showed Ptl concentrations below the cutoff. An 8-day-old patient with C21OHD whose birth weight was 1628 g had a lower Ptl value
than the cutoff (Fig. 2A, arrow). Ptl/THEs differentiated C21OHD and PORD from TH17OHP and control with 100% (95.1%–100%) diagnostic sensitivity and 100% (99.8%–100%) diagnostic specificity with the 0.020 cutoff.

**DISCRIMINATION BETWEEN C21OHD AND PORD**

Fig. 3 shows the results of urinary metabolites of 17,20-lyase products (mg/g creatinine) in C21OHD and PORD. DHEA metabolites were 97 (11–505) in C21OHD and 4.0 (0.15–17) in PORD. Androgen metabolites were, for boys, 1.1 (0.18–36) in C21OHD and 0.19 (0.17–0.41) in PORD, and for girls, 1.1 (0.26–9.9) in C21OHD and 0.16 (0.016–1.4) in PORD. 11OHAD4 metabolite was 3.0 (0.61–24) in C21OHD and 0.077 (0.022–0.22) in PORD. All metabolites of 17,20-lyase products showed significant differences between C21OHD and PORD (DHEA metabolites, \( P < 0.001 \); androgen metabolites (male), \( P = 0.006 \); androgen metabolites (female), \( P = 0.039 \); 11OHAD4 metabolite, \( P < 0.001 \)). 11OHAD4 metabolite (11HA) discriminated between C21OHD and PORD with 100% (94.2%–100%) diagnostic sensitivity and 100% (81.4%–100%) diagnostic specificity with the 0.35 mg/g creatinine cutoff. Urinary DHEA and androgen metabolites showed overlap between C21OHD and PORD.

**Discussion**

We established a 2-step biochemical differential diagnosis for C21OHD and PORD by urinary steroid profile. First, by using a specific cutoff of the ratio of Ptl to THEs, we were able to differentiate C21OHD and PORD from TH17OHP and controls. Second, by using a specific cutoff of 11HA, we were able to distinguish between C21OHD and PORD. Although a distinctive steroid excretion pattern in C21OHD and PORD had been reported (3, 5, 6), no clear cutoff of urinary steroid metabolites was reported in any ages to discriminate between C21OHD and PORD. To the best of our knowledge, this is the first report of a cutoff for biochemical differential diagnosis of C21OHD and PORD in infants. As for TH17OHP, an adequate observation period and timing for repeat blood test are as yet unknown. It is difficult to differentiate between C21OHD and PORD by current standard hormonal testing and clinical observation (3, 4). This 2-step method can diagnose TH17OHP, C21OHD, and PORD by 1 as-

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**Fig. 3. Urinary metabolites of 17,20-lyase products DHEA (A), androgen (male) (B), androgen (female) (C), and 11OHAD4 (D) in infants with C21OHD and PORD.**

Dashed line in (D) shows cutoff of 0.35 mg/g creatinine for 11OHAD4 metabolite. ▲, C21OHD; ●, PORD.
say with a noninvasive spot urine sample while infants have increased concentrations of serum 17OHP.

Increased Ptl and Ptl/THEs in C21OHD and PORD must reflect the impaired activity of 21-hydroxylase. We showed that a specific cutoff of Ptl/THEs differentiated C21OHD and PORD from TH17OHP and controls with no overlap. In patients with C21OHD, impaired activity of 21-hydroxylase led to an increase in the precursor steroid 17OHP. As a result, Ptl, which is a metabolite of 21DOF, was increased. Because the activity of 21-hydroxylase in patients with PORD was impaired by less electron supply from POR, increased Ptl was reasonable.

Decreased 11HA in PORD must reflect the impaired activity of 17,20-lyase. The reason is unclear why the urinary 11OHA4 metabolite was the only 1 among other metabolites of 17,20-lyase products that showed a difference between C21OHD and PORD, but the reason is probably that 11OHA4 is of purely adrenal origin. DHEA metabolites showed tiny overlap between C21OHD and PORD that may derive from unexpectedly low DHEA metabolites in 3 patients with C21OHD. On the 1 hand, androgen metabolites showed a clear overlap that can be explained by androgen production in the backdoor pathway (Fig. 1, dashed arrow), which is proven in the tammar wallaby (12) and postulated in the human newborn (13). Androsterone can be derived not only from AD4 and dihydrotestosterone in the conventional pathway but also from 5α-pregnan-3α,17α-diol-20-one in the backdoor pathway (12, 13). In PORD, androsterone could increase during early infancy (13), resulting in overlap in androgen metabolites.

One may argue that PD, the ratio of PD to 17,20-lyase metabolites (e.g., 11HA, DHEA metabolites), or the ratio of 17α-hydroxyprogrenenolone metabolite to DHEA metabolites (13) is discriminatory (Fig. 1). Unfortunately, the measurement of PD is sometimes problematic in our GC-MS method in newborns and we cannot calculate the concentration or the ratio. We have reported that the ratio of 17α-hydroxyprogrenenolone metabolites to DHEA metabolites discriminated 22 patients with PORD from healthy infants (13), whereas this ratio could not discriminate C21OHD and PORD completely in this cohort (data not shown).

This study had 4 limitations. First, nonclassic 21OHD was not included in study subjects. Nonclassic 21OHD is known to have lower blood 17OHP than C21OHD (14, 15). Thus, it is conceivable that these individuals have lower Ptl/THEs and 11HA than C21OHD. Further studies are required to determine if the same cutoff can discriminate between nonclassic 21OHD and PORD. Second, the total number of affected infants, and specifically the number of very-low-birthweight infants (only 2), was limited in this study. Infants born with very low birthweight should have less hepatic enzyme activities such as 5β-reductase, 3α-hydroxysteroid dehydrogenase, and 20α-hydroxysteroid dehydrogenase, leading to less conversion of 21DOF to Ptl. Indeed, 1 patient with C21OHD who was born with low birthweight had a lower Ptl value than the cutoff. The third limitation is the random time at which urine samples were collected, although it is known that steroid metabolism starts to show diurnal variation around 2–3 months of age (16). We unintentionally proved that we could use the urine sample whenever it is collected. As for the fourth limitation, our data in Japanese infants may not apply to other ethnic populations. Enzymatic residual activity in PORD has been reported to differ depending on genotype (17). The common mutation was R457H in Japanese PORD (7, 18) and A287P in whites (19). R457H has 1%–3% supporting activity for 17α-hydroxylase and virtually no activity for 17,20-lyase compared to wild type, whereas A287P has 40% activity for 17α-hydroxylase and about 20% for 17,20-lyase (17, 19, 20). In fact, all subjects with PORD were Japanese with homozygous or heterozygous R457H mutation (Table 2). For PORD cohorts with higher 17,20-lyase activity (i.e., A287P), 11HA might not be as useful. Thus other cutoffs may be required for non-Japanese.

In conclusion, we demonstrated a 2-step biochemical differential diagnosis for C21OHD and PORD by urinary steroid metabolites such as Ptl, THEs, and 11HA. We believe that this 2-step biochemical diagnosis would be valuable for Japanese infants whose clinical differential diagnosis is difficult.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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References