C16 Hydroxylation of 3β-Hydroxy-Δ5-steroids during the Early Neonatal Period

Noriko Tagawa, Satoshi Kusuda, and Yoshiharu Kobayashi

Clinical Chemistry Laboratory, Kobe Pharmaceutical University, Kobe, 658, Japan, and Department of Neonatology, Prenatal Center of Osaka City General Hospital, 2-13-22, Miyakojima-ondori, Miyakojima, Osaka, 554, Japan. Received July 10, 1997; accepted September 16, 1997.

Temporal changes of the serum levels of 16α-hydroxypregnenolone (3β,16α-dihydroxy-5-pregnen-20-one) 3-sulfate (16-OH-Preg S) and 16α-hydroxydehydroepiandrosterone (3β,16α-dihydroxy-5-androsten-17-one) 3-sulfate (16-OH-DHEA S) were investigated by analyzing the levels of their precursor steroids, pregnenolone (3β-hydroxy-5-pregnen-20-one) 3-sulfate (Preg S) and dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one) 3-sulfate (DHEA S), respectively, in the early neonatal period. The serum levels of these steroids were measured by GC-MS in full-term (gestational age: 37–41 weeks), pre-term (gestational age: 28–36 weeks) and extremely immature (gestational age: 24–27 weeks) infants. The changes in 16α-hydroxysteroid production were also investigated by analyzing the ratios of the serum levels of 16-OH-Preg S and Preg S (16-OH-Preg S/Preg S ratio), and 16-OH-DHEA S and DHEA S (16-OH-DHEA S/DHEA S ratio).

It was confirmed that the 16α-hydroxylation of DHEA S and Preg S increased after birth, and the 16-OH-Preg S/Preg S ratio in full-term infants was significantly higher than in pre-term and extremely immature infants at days 0, 1–6 and 7–13. On the other hand, there were no significant differences between the 16-OH-DHEA S/DHEA S ratios of the three groups at days 0, 1–6 or 7–13.

The mechanism of differences in the 16α-hydroxylation of Preg S and DHEA S is also discussed.

Key words: 16α-hydroxypregnenolone sulfate; 16α-hydroxydehydroepiandrosterone sulfate; serum level; GC-MS; pre-term infant; full-term infant

In the fetal liver, 3β-hydroxy-Δ5-steroids from the fetal adrenal gland actively undergo 16α-hydroxylation.1–3 One of the most important 16α-hydroxysteroids in the fetus is 16α-hydroxydehydroepiandrosterone (3β,16α-dihydroxy-5-androsten-17-one) 3-sulfate (16-OH-DHEA S), which is converted to estriol in the placenta.4–6 Dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one) 3-sulfate (DHEA S), originating from the fetal adrenal gland, is the precursor of 16-OH-DHEA S.7 The other 16α-hydroxysteroid, 16α-hydroxy pregnenolone (3β,16α-dihydroxy-5-pregnen-20-one) 3-sulfate (16-OH-Preg S), is produced from pregnenolone (3β-hydroxy-5-pregnen-20-one) 3-sulfate (Preg S) in the fetal liver.8–10 Although the levels of 16-OH-Preg S in the fetal and early neonatal circulation are nearly the same as those of 16-OH-DHEA S,11,12 the physiological significance and metabolic fate of 16-OH-Preg S have not been determined.

After birth, the fetal zone of the adrenal gland, which is the main site of Preg S and DHEA S formation, undergoes rapid involution and reorganization to the adult cortex.13 As a result, the neonatal enzyme system for steroidogenesis and the hormonal milieu of neonates are markedly different from those of adults, and the levels of 3β-hydroxy-Δ5-steroids are decreased after birth. In general, reductions in the level of the precursor steroid result in decreases in those produced. However, the serum levels of 16α-hydroxysteroids such as 16-OH-DHEA and 16-OH-Preg S remain high in the early neonatal period.12

There are two possible explanations for this phenomenon: 16α-hydroxylase activity may be retained or increased, or the supply of the precursor for estriol formation, 16-OH-DHEA S, may not be required after birth, leading to an accumulation of the final product, 16-OH-DHEAS.

This study was performed to investigate why the serum levels of 16-OH-DHEA S and 16-OH-Preg S remain high during the early neonatal period.

MATERIALS AND METHODS

Materials The materials used in this study were obtained from the following sources: 16-OH-Preg, 16-OH-DHEA, Preg and DHEA, Sigma Chemical Co., Ltd. (U.S.A.); stigmastanol (3β-hydroxy-24-ethyl-5,22-cholestadiene), GL Sciences, Inc. (Tokyo, Japan); N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), Wako Pure Chemical Industries (Osaka, Japan); arylsulfatase (EC.3.1.6.1. from Helix pomatia), Boehringer Mannheim (Mannheim, Germany); ASPEC pak® (disposable octadecyl silane cartridge, 0.9 mm i.d. × 20 mm) column, M&S Instruments Trading, Inc. (Osaka, Japan); Accell Plus QMA® (disposable quaternary methyl ammonium conjugated silica cartridge, 300 mg) column, Waters Corp. (Milford, MA, U.S.A.).

Steroid sulfate levels were determined in serum samples from 122 newborn infants. The infants were grouped as full-term (26 males include 6 deliveries by cesarean section (CS) and 5 females include 1 delivery by CS), pre-term (44 males include 18 deliveries by CS and 24 females include 10 deliveries by CS) or extremely immature pre-term (12 males include 6 deliveries by CS and 11 females include 6 deliveries by CS) based on birth between 37–41, 28–36 and 22–27 weeks of gestation, respectively. Infants with endocrine disease complications were excluded from this study. Serum samples were obtained by venipuncture at days 0, 1–6 and 7–13. Samples were kept frozen at −40°C until being assayed.

HPLC and GC-MS Conditions HPLC was performed on a Waters 6000A pump, a Waters model 710B auto-
sampler (Waters Corp., Milford, MA, U.S.A.) and a Waksol-II5C18-HG 4.6 mm i.d. x 150 mm column (Wako Pure Chemical Industries, Osaka, Japan) run at 40°C, using water-acetonitrile (1:1, v/v) as the mobile phase at a flow rate of 1 ml/min. Data reduction was carried out using a Uvidec-100-IV detector (210 nm) and Waters 805 data station. Under these conditions, the retention times of the steroids were as follows: 16-OH-DHEA, 3.38 min; 16-OH-Preg, 3.84 min; DHEA, 8.31 min; Preg, 19.55 min.

GC-MS was performed on a Hewlett-Packard model 5890 gas chromatograph directly coupled with a Hewlett-Packard model 5971 mass detector (electron impact, 70 eV) equipped with a Hewlett-Packard chemstation data analyzer. The J & W DB-1 column (0.25 mm i.d. x 15 m, 0.1 μm film thickness) was run under the following conditions: helium gas at a flow rate of 1 ml/min; injection port temperature, 300°C; oven temperature, 50°C for 1.5 min followed by 50—300°C at 30°C/min and finally held at 300°C for 2 min.

**Preparation of Serum Samples for Measurement of Steroid Sulfates**

Methods for extracting steroid sulfates from 0.05 ml of serum samples and enzymatic hydrolysis were described previously. The residue after hydrolysis was dissolved in 0.2 ml of the HPLC mobile phase and 0.15 ml of the sample was applied to HPLC. The eluates between 3.0 to 5.0 min (as 16-OH-DHEA and 16-OH-Preg fraction), 8.0 to 9.5 min (DHEA), and 19.0 to 21.5 min (Preg) were collected. These three fractions were combined into a tube and 12.5 μg of stigmasterol in methanol (0.1 ml) was added as an internal standard. After evaporation, TMS (trimethylsilyl) derivatization was performed at 120°C for 30 min with 0.1 ml of BSTFA (bis(trimethylsilyl)-trifluoroacetamide) and the reaction mixture was evaporated to dryness. The residue was dissolved in 0.1 ml of dichloromethane, and 2 μl of this sample was subjected to GC-MS analysis. Each steroid was measured by selected ion monitoring (Preg-TMS, m/e 388 [M]⁺; DHEA-TMS, m/e 360 [M]⁺; 16-OH-DHEA-TMS, m/e 304 [M—15—129]⁺; 16-OH-Preg-TMS, m/e 386 [M—90]⁺; stigmasterol-TMS, m/e 484 [M]⁺).

**GC-MS Calibration Curve and Measurement of Each Steroid**

Serially diluted authentic steroid solutions were dissolved in methanol and were added to tubes containing 12.5 μg of stigmasterol in methanol (0.1 ml) as an internal standard. The solvent was evaporated in vacuo. The trimethylsilylation and GC-MS conditions were the same as those described above. The steroid levels were measured by comparing the obtained peak area from each steroid in the sample and the authentic standard to that obtained from the internal standard. The serum levels of each steroid sulfate were corrected according to their recoveries (87.7—95.5%).

**Statistical Analysis**

Values are expressed as the means ± S.D. Differences between the two groups were statistically analyzed by the Mann-Whitney U-test. A p value below 0.05 was considered significant.

**RESULTS**

**Serum Levels of 16-Hydroxysteroids and Their Precursors**

The intra- and inter-assay coefficients of variation, as obtained by multiple determinations of serum sample for each steroid sulfate, were 2.3—9.3% and 7.3—13.1%, respectively. The time course of changes in serum levels of 16-OH-Preg S, 16-OH-DHEA S, Preg S and DHEA S in neonates are shown in Figs. 1 and 2.

**Fig. 1. Temporal Changes in Serum Levels of 16-OH-Preg S and 16-OH-DHEA S during the Early Neonatal Period**

(a), (d): Full-term infants (gestational age: 37—41 weeks); (b), (e): pre-term infants (gestational age: 28—36 weeks); (c), (f): extremely immature infants (gestational age: 22—27 weeks). The numbers of infants are shown in parentheses. *p < 0.05, **p < 0.01, ***p < 0.001, N.S.: not significant.

Serum 16-OH-Preg S Levels (Fig. 1(a), (b), (c)): The mean values of the serum 16-OH-Preg S level in full-term infants (gestational age: 37—41 weeks, n = 14, Fig. 1(a)) at day 0 was 2.4 ± 1.6 μg/ml, and this level was retained from days 7—13. The level at day 0 in pre-term infants (gestational age: 28—36 weeks, n = 40, Fig. 1(b)) was 1.7 ± 1.3 μg/ml and that in extremely immature infants (22—27 weeks, n = 14, Fig. 1(c)) was 1.4 ± 1.0 μg/ml. In both premature groups, these values were increased four- or five-fold from days 1—6, and the differences between day 0 and from days 1—6 were significant (pre-term: p < 0.001, extremely immature: p < 0.01). Moreover, these levels from days 7—13 were significantly higher than those on day 0 (pre-term: p < 0.001; extremely immature: p < 0.01).
There were no significant differences between the serum 16-OH-Preg S levels in the three groups at day 0. However, from days 1—6, the levels in pre-term infants were significantly higher than those in the full-term group ($p < 0.05$).

Serum 16-OH-DHEA S Levels (Fig. 1(d), (e), (f)): At day 0, the mean levels of 16-OH-DHEA S in the full-term, pre-term and extremely immature infants were $1.2 \pm 0.8 \mu g/ml$ ($n=16$, Fig. 1(d)), $1.5 \pm 1.8 \mu g/ml$ ($n=40$, Fig. 1(e)) and $2.6 \pm 2.0 \mu g/ml$ ($n=14$, Fig. 1(f)), respectively. In full-term infants, no significant changes were observed between the levels at days 0, 1—6 and 7—13. In pre-term and extremely immature infants, the serum levels of 16-OH-DHEA S were increased significantly from days 1—6 in comparison to these at day 0 ($p < 0.05$).

At day 0, the level in extremely immature infants was significantly higher than that in the full-term group ($p < 0.01$), and from days 1—6, the levels in pre-term and extremely immature infants were significantly higher than in the full-term group (pre-term: $p < 0.05$, extremely immature: $p < 0.05$).

Serum Preg S (Fig. 2(a), (b), (c)) and DHEA S Levels (Fig. 2(d), (e), (f)): The levels of Preg S and DHEA S in full-term infants at days 1—6 were reduced to one quarter of those at day 0 (Preg S: $p < 0.01$, Fig. 2(a); DHEA S: $p < 0.001$, Fig. 2(d)). These steroid levels at day 0 in the pre-term group were reduced from days 7—13 (Preg S: $p < 0.05$, Fig. 2(b); DHEA S: $p < 0.01$, Fig. 2(e)). However, there were no significant decreases in the levels of either of these steroids between day 0 and days 7—13 in extremely immature infants (Fig. 2(c); Fig. 2(f)).

The levels of Preg S and DHEA S at day 0 were not different among the three groups. From days 1—6 and 7—13, Preg S levels in pre-term and extremely immature infants were significantly higher than those in full-term infants (pre-term: $p < 0.001$, extremely immature: $p < 0.01$) and DHEA S levels were also significantly higher than those in the full-term group (pre-term: $p < 0.001$, extremely immature: $p < 0.05$).
DISCUSSION

Fetal liver is a constituent of the feto-placental unit and is the site of active 16-hydroxylolation of the fetal C19 and C21 steroids from early gestation. The fetal liver produces 16-OH-DHEA S and 16-OH-Preg S from fetal adrenal DHEA S and Preg S, respectively, and these are predominant in the fetal and early neonatal circulation. Likewise, 3β-hydroxy-Δ4-steroids originating from the fetal adrenal gland, 16-OH-DHEA S, are important as a precursor of estriol. Although the levels of 16-OH-Preg S in the serum of neonates are nearly the same as those of 16-OH-DHEA S, the physiological significance and metabolic fate of the former remain unclear. Our previous studies on 16-dehydroepiandrosterone (3β-hydroxy-5β pregnenadien-20-one) 3-sulfate (16-DHP S) in neonatal serum suggested that 16-OH-Preg S is a precursor of the 16-DHP S. On the other hand, Jaffé et al. reported that 16-OH-Preg may be a precursor of 16-OH-DHEA. However, Godtha et al. observed no formation of metabolites in an incubation medium containing fetal adrenal homogenate and 14C-labeled 16-OH-Preg, although they confirmed that 16-OH-Preg was converted to 16-hydroxyprogesterone (16α-hydroxy-4-pregnen-3,20-dione) under the same conditions, but using instead a placental homogenate.

Serum levels of DHEA S and Preg S are considered to influence those of 16-OH-DHEA S and 16-OH-Preg S, respectively. So, we investigated the correlation between the serum levels of these 16-hydroxysteroids and those of their precursors. The serum levels of 16-OH-DHEA S and 16-OH-Preg S in pre-term and extremely immature infants were significantly increased from days 1—6 relative to those at day 0 (Fig. 1 (b), (c), (e), (f)). These levels in full-term infants did not change from day 0 to 13 (Fig. 1 (a), (d)). On the other hand, there were no changes in the serum levels of the precursors DHEA S and Preg S, in pre-term and extremely immature infants, between day 0 and 1—6 (Fig. 2 (b), (c), (e), (f)), while those in full-term infants were significantly decreased (Fig. 2 (a), (d)). Thus, the changes in the serum levels of 16-OH-DHEA S and 16-OH-Preg S did not parallel those of their precursors. Therefore, we examined the quantitative relationships between 16-hydroxysteroids and their precursors by analyzing the ratios of the levels of 16-OH-DHEA S or 16-OH-Preg S and their precursors.

Fig. 3 shows that the 16-OH-Preg S/Preg S and 16-OH-DHEA S/DHEA S ratio in each group at day 0 significantly increased from days 1—6. Moreover, the 16-OH-Preg S/Preg S ratios tended to be elevated significantly until days 7—13 relative to those on day 0 in the three groups. These results could be accounted for by the increase of 16-hydroxylase activity or the accumulation of 16-OH-Preg S and 16-OH-DHEA S after birth. Reynolds reported that 16-hydroxylase activities in the liver of a 2.5-d-old infant were significantly higher than those of a 2.5-month-old infant. Further, the neonatal urinary excretion of conjugated 16-OH-Preg and 16-OH-DHEA was prominent during the neonatal period. These findings suggest that the increase of the 16-OH-Preg S/Preg S and 16-OH-DHEA S/DHEA S ratio during the
early neonatal period is due to the increase of 16-hydroxylase activity rather than to the accumulation of 16-hydroxysteroids.

As shown in Fig. 4 (a) and (b), the 16-OH-Preg S/Preg S ratio in full-term infants at days 0, 1—6 and 7—13 was higher than those in pre-term and extremely immature infants. However, the 16-OH-DHEA S/DHEA S ratio was not different between the three groups. Moreover, the ratio of the serum level of 16-OH-Preg S against those of its precursor, Preg S, was significantly changed according to gestational age. These results suggest that the mechanism of regulation of the production of 16-OH-Preg S is different from that of 16-OH-DHEA S. There are many lines of evidence regarding the regulation of steroid production by steroids in vivo and in vitro.  

Sano et al. 28) reported that the 16-hydroxylase system in human fetal liver is inhibited by DHEA, Preg and that their sulfates, and their inhibitory effects on 16-hydroxylation towards Preg are stronger than those towards DHEA. These results support our observation that there were no changes in the 16-OH-DHEA S/DHEA S ratio according to gestational age. Moreover, Preg S levels in pre-term and extremely immature infants were significantly higher than those in full-term infants (Fig. 2 (a), (b), (c)). Again, Sano’s data explain our observation that the 16-OH-Preg S/Preg S ratio in full-term infants was significantly higher than that in pre-term or extremely immature infants.

In conclusion, our results suggest that the regulation of the production of 16-OH-Preg S during the early neonatal period is different from that for 16-OH-DHEA S, and that the production of 16-OH-Preg S may be regulated by fetal adrenal 3β-hydroxy-Δ5-steroids.

Further studies of the inhibition of 16-hydroxylation of Preg S in fetal and neonatal liver and the physiological significance of 16-OH-Preg S are currently under way in our laboratory.

Acknowledgements This work was supported in part by Kobe Pharmaceutical University Collaboration Fund and The Science Research Promotion Fund of The Japan Private School Promotion Foundation.

REFERENCES