16-Dehydroprogrenenolone 3-Sulfate, Its Source and Metabolism in the Feto-Placental Unit

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We have investigated the serum concentration of 16-dehydroprogrenenolone (3β-hydroxy-5,16-pregadien-20-one) 3-sulfate (16-DHPS) in the umbilical artery (U.A.), umbilical vein (U.V.) and maternal vein (M.V.) to discover the origin of 16-DHPS. Although there was no significant difference between the levels of 16-DHPS in U.A. (18±15 ng/ml, mean±S.D., n=28) and U.V. (10±9 ng/ml, n=28), these values were significantly higher (U.A., p<0.001; U.V., p<0.001) than that in M.V. (2±3 ng/ml, n=28). These levels in the U.A. and U.V. did not fall in infants (30±18 ng/ml, n=7) during the early neonatal period (2–7 d after birth). A significant correlation between the serum levels of 16-DHPS and 16-hydroxyprogrenenolone (3β,16α-dihydroxy-5-pregnen-20-one) 3-sulfate (16-OH-PregS), which may be the precursor steroid for 16-DHPS, was observed in the U.A. (r=0.630, n=28, p<0.001), but not in the U.V. Moreover, this significant correlation persisted during the early neonatal period (p<0.05, r=0.842, n=7), although the neonate had been separated from the maternal milieu. These results suggest that 16-DHPS originates in the fetus.

To confirm the metabolic pathway of 16-DHPS (i.e. pregnenolone (3β-hydroxy-5-pregnen-20-one) 3-sulfate (PregS) → 16-OH-PregS → 16-DHPS), we investigated the correlation between the serum concentrations of the precursor steroid and the product in both the U.A. and U.V. A significant correlation was obtained between the serum concentrations of PregS and 16-OH-PregS both in the U.A. (p<0.001, r=0.563, n=28) and U.V. (p<0.05, r=0.476, n=27). As described above, the serum levels of 16-DHPS and 16-OH-PregS only correlated significantly in the U.A. These findings support the existence of the pathway, PregS → 16-OH-PregS → 16-DHPS, in the fetus.

Key words 16-dehydroprogrenenolone 3-sulfate; metabolism; serum level; fetus; neonate; GC-MS

We have identified 16-dehydroprogrenenolone (3β-hydroxy-5, 16-pregadien-20-one, 16-DHP) in the serum of immature infants and suggested it is a form of sulfate conjugate.11 This steroid was initially identified in the mono-sulfate conjugate steroid fraction of neonatal feces by Gustafsson et al.12 Subsequently, 16-DHP was also identified in human placenta,3 urine,4–7 gall bladder bile,8 meconium9 and amniotic fluid.10

Reynolds et al.13 suggested that the double bond between C-16 and 17 in the steroid skeleton is introduced in the placenta, based on their finding that 16-hydroxyprogestrone (16α-hydroxy-4-pregnen-3, 20-dione) was converted to 16-hydroxyprogestrone (4,16-pregadiene-3, 20-dione) following incubation with human placenta. Smith et al.14 also identified 16-DHP in incubation medium containing 17-hydroxyprogrenenolone (3β,17α-dihydroxy-5-pregnen-20-one, 17-OH-Preg) as a precursor and human placenta. On the other hand, Huhtaniemi15 showed that 16-DHP was formed from pregnenolone (3β-hydroxy-5-pregnen-20-one, Preg) by the fetal liver in vitro and suggested that 16-hydroxyprogrenenolone (3β,16α-dihydroxy-5-pregnen-20-one, 16-OH-Preg) produced in liver from Preg was the precursor of 16-DHP. In our previous paper,16 we proposed that 16-DHP 3-sulfate (16-DHPS) was formed in the fetus and/or neonate rather than in the placenta in vivo from an investigation of its serum concentrations.

To obtain a complete understanding of the formation of 16-DHPS in the feto-placental unit, we have investigated serum levels of 16-DHPS and its precursor steroids such as pregnenolone 3-sulfate (PregS) or 16-OH-Preg 3-sulfate (16-OH-PregS), in the umbilical artery (U.A.), umbilical vein (U.V.) and maternal peripheral vein (M.V.).

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MATERIALS AND METHODS

Materials The chemicals used in this study were obtained from the following sources: 16-DHP (Steraloids Inc., Newport, RI, U.S.A.); 16-OH-Preg, 16-hydroxydehydroepiandrosterone (3β,16α-dihydroxy-5-androsten-17-one) 3-sulfate (16-OH-DHEAS), Preg and dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one, DHEA) (Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.); 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol) (GL Sciences Inc., Tokyo, Japan); N-O-bis(3trimethylsilyl)trifluoroacetamide (BSTFA) (Wako Pure Chemical Industries, Osaka, Japan); aroylulfsulfate (EC 3.1.6.1.1. from Helix pomatia) (Boehringer Mannheim, Mannheim, Germany); Sep-Pak Vac TLC18 (disposable octadecyl silane cartridge, 500 mg) and Accell Plus QMA19 (disposable quaternary methyl ammonium conjugated silica cartridge, 300 mg) (Waters Corp., Milford, MA, U.S.A.). 16-DHPS was synthesized in this laboratory from 16-DHP as described by Maeda.20 Water was purified with a Milli-Q system (Nippon Millipore Ltd., Tokyo, Japan) and other chemicals were of analytical grade.

Serum samples from the U.A. (n=28), U.V. (n=28) and M.V. (n=28) were obtained from pregnant women who delivered at term (37–42 weeks of gestation). Cases with delivery complications were excluded from the study. Serum samples taken from normal infants (2–7 d after birth, n=7) were also used. The samples were kept frozen at −20°C until assayed.

HPLC and GC-MS Conditions HPLC was performed using a Waters 6000A pump, a Waters model 710B autosampler (Waters Corp.) and a Wako-2 5C18-HG 150 mm×4.6 mm i.d. column (Wako Pure Chemical Industries) ran at
40°C. The separations were performed at a flow rate of 1 ml/min with a linear gradient of acetonitrile (2%/min) from 30 to 70% in water. 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, 8.6 min; 16-OH-Preg, 10.2 min; 17-OH-Preg, 15.4 min; DHEA, 16.2 min; 16-DHP, 20.9 min; and Preg, 21.5 min.

GC-MS was performed on a Hewlett-Packard model 5890 gas chromatograph equipped with a Hewlett-Packard model 5971 mass detector (electron impact, 70 eV). The J & W DB-1 column, 15 m × 0.25 mm i.d., 0.1 μm film thickness, was run under the following conditions: 1 ml/min helium gas flow rate; injection port temperature, 300°C; oven temperature, 50°C for 0.5 min followed by 50–300°C at 30°C/min and, finally, held at 300°C for 2 min.

Preparation of Serum Samples and GC-MS Measurement of Steroid Sulfates Extraction of steroid sulfates from serum samples and enzymatic hydrolysis of the sulfates were carried out by the reported methods. In short, 0.05 ml (infants) or 0.2 ml (U.A., U.V. and M.V.) serum samples were diluted with 1 ml 0.9% NaCl and applied to a SepPak Vac tC18. After washing with water, the Accell Plus QMA® was connected to the Sep-Pak Vac tC18® outlet. The combined cartridges were flushed with 72% aqueous ethanol so that the unconjugated steroids retained in the Sep-Pak Vac tC18® were discarded, then the Sep-Pak Vac tC18® was disconnected. After steroid glucuronides were removed by flushing with 0.3 m acetic acid in 72% aqueous ethanol, steroid mono-sulfates were obtained with 0.3 m sodium acetate-acetic acid in 72% aqueous ethanol (pH 6.3). The eluate was evaporated in vacuo, and the residue was hydrolyzed with arylsulfatase in 0.25 m acetate-sodium acetate buffer (pH 6.0). Steroids were extracted with dichloromethane and then evaporated in vacuo. The residue was dissolved in 0.2 ml HPLC mobile phase, water-acetonitrile (70:30, v/v), and 0.15 ml of the sample was subjected to HPLC. The eluates between 7.0 to 11.0 min (16-OH-DHEA and 16-OH-Preg, Fraction 1), 14.8 to 18.0 min (17-OH-Preg and DHEA, Fraction 2), and 20.3 to 23.5 min (16-DHP and Preg, Fraction 3) were collected. Fraction 1 and 2 (16-OH-DHEA, 16-OH-Preg, 17-OH-Preg and DHEA) were combined in one tube. Fraction 3 (16-DHP and Preg) was analyzed separately from the other steroids to avoid artificial formation of 16-DHP from 16-OH-Preg. Stigmasterol in methanol (12.5 ng/0.1 ml) was added to each sample as an internal standard. After evaporation, trimethylsilyl (TMS) derivatization was performed with 0.1 ml BSTFA at 120°C for 30 min. Under these derivatization conditions, no dehydrated or decomposed compounds were observed. The reaction mixture was evaporated to dryness. The residue was dissolved in 0.1 ml dichloromethane and 2 μl was analyzed by GC-MS as described above. Each steroid was measured by the following selected ion monitor: 16-DHP-TMS, m/z 386 [M]+; Preg-TMS, m/z 388 [M]+; DHEA-TMS, m/z 360 [M]+; 16-OH-DHEA-TMS, m/z 304 [M−15−129]+; 16-OH-Preg-TMS, m/z 386 [M−90]+; 17-OH-Preg-TMS, m/z 299 [M−15−90]; stigmaster-TMS, m/z 484 [M]+. The results of the GC-MS measurement were expressed as serum steroid sulfate values. Overall recoveries were monitored using serum samples with added known amounts of authentic steroids. The results were as follows: 16-DHPS, 95.7±3.4% (50 ng/ml, n=6); PregS, 87.2±4.3% (500 ng/ml, n=5); and DHEAS, 91.3±4.4% (500 ng/ml, n=5). The measurable range for each steroid was as follows: 16-DHPS, 1.5–753 ng/ml; 16-OH-PregS, 12–5957 ng/ml; PregS, 12–3008 ng/ml; 17-OH-PregS, 12–2978 ng/ml; DHEAS, 12–6133 ng/ml; and 16-OH-DHEAS, 12–6221 ng/ml. The coefficient of variation for the intra- and inter-assay of each steroid was 2.3−4.7% (n=5) and 5.4−8.2%, respectively.

Statistical Analysis Values are expressed as means ±S.D. Differences between two groups were statistically analyzed by the Mann-Whitney U-test. A simple correlation coefficient between the concentrations of steroid sulfates in the same serum sample was obtained using the Pearson correlation coefficient. A p value below 0.05 was considered significant.

RESULTS

Serum Concentrations of 16-DHPS and Other Steroid Sulfates in the U.A., U.V. and M.V. To investigate the source of 16-DHPS, we compared the serum concentrations of 16-DHPS in the U.A. (n=28), U.V. (n=28) and M.V. (n=28) of pregnant women who delivered at term (37−42 weeks of gestation). Serum levels of other steroid sulfates (16-OH-PregS, 16-OH-DHEAS, PregS, 17-OH-PregS, DHEAS) were also measured in the same serum samples (Fig. 1).
Although there was no significant difference between the levels of 16-DHPS in the U.A. and U.V., the values in the U.A. and U.V. were significantly higher (U.A., \( p < 0.001 \); U.V., \( p < 0.001 \)) than in the M.V. The concentration of other steroid sulfates measured in this study did not differ significantly between the U.A. and U.V., but the levels in the U.A. and the U.V. were also significantly higher than in the M.V. (Fig. 1).

**Comparison of Serum Levels of 16-DHPS and 16-OH-PregS in Infants (2—7d after Birth)** To investigate the contribution of the maternal environment (e.g., placenta) to the formation of 16-DHPS, we investigated the serum concentration of 16-DHPS and 16-OH-PregS in infants.

The 16-DHPS and 16-OH-PregS levels in 2—7-d-old infants were 30 ± 18 ng/ml (mean ± S.D.; \( n = 7 \)) and 2444 ± 1384 ng/ml (\( n = 7 \)), respectively. The serum concentrations of 16-DHPS correlated significantly with those of 16-OH-PregS in individual infants (\( p < 0.05, r = 0.842 \)).

**Investigation of the Metabolic Pathway of Steroid Sulfates: Approaches Based on the Correlation between Serum Levels of Precursor Steroids and Their Products in the U.A. and U.V.**

a) Established Metabolic Pathway of Steroid Sulfates: PregS → 17-OH-PregS → DHEAS → 16-OH-DHEAS: The relationship between the serum levels of precursor steroid sulfates and their products in the U.A. and U.V. are shown in Table 1. The levels of PregS, 17-OH-PregS and DHEAS correlated significantly both in the U.A. (\( p < 0.001, r = 0.643, 0.608 \) and 0.596 for PregS; 17-OH-PregS; 17-OH-PregS; DHEAS; and PregS: DHEAS, respectively) and U.V. (\( p < 0.001, r = 0.753, 0.643 \) and 0.651 for PregS: 17-OH-PregS; 17-OH-PregS: DHEAS; and PregS: DHEAS, respectively). The levels of DHEAS correlated significantly with those of 16-OH-DHEAS in the U.A. (\( p < 0.001, r = 0.652 \)), but not in the U.V. These results support the existence of the established metabolic pathway above.

b) Proposed Metabolic Pathway of 16-DHPS: PregS → 16-OH-PregS → 16-DHPS: A significant correlation for the precursor steroid and product was observed between each step of the pathway in the U.A. (\( p < 0.001, r = 0.563, 0.630 \) and 0.622 for PregS: 16-OH-PregS; 16-OH-PregS: 16-DHPS; and 16-OH-PregS: 16-DHPS, respectively) but there was only a weak correlation between the serum concentration of PregS and 16-OH-PregS (\( p < 0.05, r = 0.476 \)), and no correlation between that of 16-OH-PregS and 16-DHPS in the U.V. (Table 1).

**Serum Levels of 16-DHPS in Adults** Serum 16-DHPS levels of normal men (age: 22—38 years, \( n = 9 \) and 49—65 years, \( n = 9 \)) and non-pregnant normal women (age: 22—34 years, \( n = 8 \) and 55—63 years, \( n = 8 \)) were determined as described in the Methods section (sample volume: 0.2 ml). However, 16-DHPS was not detected in serum from normal adults by the present assay system (data not shown).

**DISCUSSION**

In this study we found that the serum levels of 16-DHPS were significantly higher in the U.A. and U.V. than in the M.V. (Fig. 1). We also observed that there was no fall in the serum concentration of 16-DHPS in infants (2—7 d after birth) compared with the U.A. or U.V., although the neonates were no longer in the maternal milieu (Fig. 1). These results may be explained by our previous finding that the 16-hydroxylation of Preg and DHEA increases temporarily after birth.55 Moreover, the significant correlation between the serum 16-DHPS and 16-OH-PregS levels in the U.A. (\( p < 0.001, r = 0.630, n = 28 \)) persisted in infants during the early neonatal period (2—7 d after birth, \( p < 0.05, r = 0.842, n = 7 \)). These results suggest that the origin of 16-DHPS is the fetus.

No differences in the serum concentrations of the steroid sulfates examined in this study, 16-DHPS, 16-OH-PregS, 16-OH-DHEAS, PregS, 17-OH-PregS and DHEAS, were ob-

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**Table 1. Correlation between Serum Levels of 16-DHPS and Other Steroid Sulfates in the U.A. and U.V.**

<table>
<thead>
<tr>
<th></th>
<th>PregS</th>
<th>16-OH-PregS</th>
<th>17-OH-PregS</th>
<th>DHEAS</th>
<th>16-OH-DHEAS</th>
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<td>U.A.</td>
<td></td>
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<tr>
<td>16-DHPS</td>
<td>0.622***</td>
<td>0.630***</td>
<td>0.391NS</td>
<td>0.297NS</td>
<td>0.398NS</td>
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<tr>
<td></td>
<td>(28)</td>
<td>(28)</td>
<td>(24)</td>
<td>(24)</td>
<td>(24)</td>
</tr>
<tr>
<td>PregS</td>
<td>0.563**</td>
<td>0.643***</td>
<td>0.596***</td>
<td>0.472*</td>
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<tr>
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<td>(28)</td>
<td>(28)</td>
<td>(28)</td>
<td>(27)</td>
<td></td>
</tr>
<tr>
<td>16-OH-PregS</td>
<td>0.383NS</td>
<td>0.501*</td>
<td>0.645***</td>
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<td></td>
<td>(23)</td>
<td>(23)</td>
<td>(23)</td>
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<tr>
<td>17-OH-PregS</td>
<td>0.608NS</td>
<td>0.254NS</td>
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<td></td>
<td>(28)</td>
<td>(23)</td>
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<tr>
<td>DHEAS</td>
<td>0.652***</td>
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<td>(23)</td>
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<td>U.V.</td>
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<tr>
<td>16-DHPS</td>
<td>0.194NS</td>
<td>0.201NS</td>
<td>0.175NS</td>
<td>0.019NS</td>
<td>0.232NS</td>
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<td></td>
<td>(28)</td>
<td>(28)</td>
<td>(24)</td>
<td>(25)</td>
<td>(23)</td>
</tr>
<tr>
<td>PregS</td>
<td>0.476*</td>
<td>0.753***</td>
<td>0.651***</td>
<td></td>
<td>0.329NS</td>
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<td>(27)</td>
<td>(28)</td>
<td>(29)</td>
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<td>(23)</td>
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<tr>
<td>16-OH-PregS</td>
<td>0.370NS</td>
<td>0.341NS</td>
<td>0.570**</td>
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<td>(24)</td>
<td>(21)</td>
<td>(23)</td>
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<tr>
<td>17-OH-PregS</td>
<td>0.643***</td>
<td>0.021NS</td>
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<td></td>
<td>(26)</td>
<td>(24)</td>
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<tr>
<td>DHEAS</td>
<td>0.360NS</td>
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<td>(23)</td>
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Values give the correlation coefficient. Number of data points is given in parenthesis. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \). N.S.: not significant.
served between the U.A. and U.V. It has been reported that all these steroids, except 16-DHPS, are predominantly secreted from the fetus.\(^{19}\) If the steroids are produced in the fetus, it seems reasonable to assume that their serum levels would be higher in the U.A. than in the U.V. However, this is not necessarily the case because of their slow metabolic clearance rate.\(^{17,18}\)

It has been established that the metabolic pathway of 16-OH-DHEAS from PregS in the fetus is PregS→17-OH-PregS→DHEAS→16-OH-DHEAS.\(^{19}\) To confirm the presence of this pathway, we investigated the correlation between the serum levels of the precursor steroid sulfates and their products both in the U.A. and U.V. (Fig. 2). Significant correlations were obtained between each of the above pathway both in the U.A. \((p<0.01, r=0.643, 0.608, 0.596\) and 0.652 for PregS; 17-OH-PregS; 17-OH-PregS: DHEAS; PregS: DHEAS; and DHEAS: 16-OH-DHEAS, respectively) and in the U.V. \((p<0.01, r=0.753, 0.643 \) and 0.651 for PregS; 17-OH-PregS; 17-OH-PregS: DHEAS and PregS: DHEAS, respectively), except for the step DHEAS→16-OH-DHEAS in the U.V. These results indicate that this metabolic pathway operates in the fetus. Our results also showed that the existence, but not the sequence, of the metabolic pathway could be confirmed by comparison of serum levels of precursor steroid and product.

Huhtaniemi\(^{11}\) reported that Preg was converted to 16-DHP in human fetal liver in vitro. Furthermore, Calvin et al.\(^{20}\) presented evidence for the formation of 16-dehydroprogesterone and 16-dehydroprogrenalone (3β-hydroxy-16-pregn-20-one) following 16-hydroxyprogesterone administration either orally or intravenously in humans. Moreover, it has been shown that in the human fetal adrenal cortex, Preg is converted to 16-OH-Preg in vitro,\(^{21}\) and PregS is converted to 16-OH-PregS in vitro in human fetal liver.\(^{12}\) These findings lead us to believe that the precursor of 16-OH-DHEAS is 16-OH-PregS which is derived from PregS in the liver or adrenal cortex of the fetus or neonate. To confirm the presence of the proposed metabolic pathway, PregS→16-OH-PregS→16-DHPS, we studied the correlation between serum levels of precursor steroids and their products, both in the U.A. and U.V. As shown in Fig. 2, the serum concentration of 16-DHPS significantly correlated with those of PregS and 16-OH-PregS in the U.A. \((p<0.001, r=0.622, n=28\) for PregS; 16-DHPS; \(p<0.01, r=0.563, n=28\) for PregS; 16-OH-PregS; \(p<0.001, r=0.630, n=28\) for 16-OH-PregS; 16-DHPS). In the U.V., however, there was only a weak correlation between the serum concentration of PregS and 16-OH-PregS \((p<0.05, r=0.476, n=27)\), and no correlation between that of 16-OH-PregS and 16-DHPS in the U.V. These results support the hypothesis that the metabolic pathway, PregS→16-OH-PregS→16-DHPS, exists in the fetus.

In conclusion, our results suggest that the origin of 16-DHPS in the fetal-placental unit is the fetus and the precursor of 16-DHPS is 16-OH-PregS which is derived from PregS.

Further studies on the metabolic fate and physiological significance of 16-DHPS in the fetus and neonate are now underway in our laboratory.

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REFERENCES