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7-HYDROXYLATION OF DEHYDROEPIANDROSTERONE IN HUMAN AMNIOTIC EPITHELIUM

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SUMMARY

The activity of 7α- and 7β-hydroxylase of dehydroepiandrosterone was demonstrated in human amniotic epithelium from the 7th to the 23rd week of pregnancy. The evidence was based on *in vitro* conversion of dehydroepiandrosterone to 7α- and 7β-hydroxydehydroepiandrosterone which were identified by paper chromatography and gas–liquid chromatography–mass spectrometry.

INTRODUCTION

The identity and content of steroids in amniotic fluid has been a subject of extensive investigations [1, 2], since it has been suggested that changes in foetal steroid metabolism may be closely reflected by steroids in amniotic fluid, and their estimation has been found to be useful in assessing the foeto-placental function. However, little attention has been paid to the possibility of further metabolism of the steroids in amniotic fluid and to the metabolizing activity of amniotic epithelium. The metabolism of [^{14}C]-labelled testosterone by cultured human amniotic fluid cells was investigated by Shanies *et al.*[3] and metabolites arising by the action of 5α-reductase and 17β-hydroxysteroid dehydrogenase were characterized. 17β-Hydroxysteroid dehydrogenase activity was also demonstrated in human amniotic epithelium abrased from the amnion from the first trimester of pregnancy [4].

We should like to report here the evidence for 7-hydroxylase activity for dehydroepiandrosterone in intact human amniotic epithelium from the 7th to the 23rd week of pregnancy.

EXPERIMENTAL

Tissue. Human amnions from the 7th to the 23rd week of pregnancy were obtained from medical abortions in 15 healthy normal women. The foetal age was calculated from menstrual data of the mother. The amnion was carefully dissected under a stereomicroscope. The epithelial layer was removed, weighed on a torsion balance and used for incubations.

Steroids. [4-^{14}C]-3β-Hydroxy-5-androsten-17-one (dehydroepiandrosterone, DHA), S.A. 52 mCi/mmol, purchased from the Radiochemical Centre, Amersham, Bucks., was purified by chromatography on silica gel precoated plates Silufol. Non-radioactive dehydroepiandrosterone was obtained from Koch–Light Labs., Colnbrook, Bucks. 3β,7α-Dihydroxy-5-androsten-17-one (7α-hydroxydehydroepiandrosterone,7α-OH-DHA) and 3β,7β-dihydroxy-5-androsten-17-one (7β-hydroxydehydroepiandrosterone,7β-OH-DHA) were prepared by acetoxylation of dehydroepiandrosterone and subsequent hydrolysis of the acetate [5].

Incubation procedure. Immediately after surgery, the amnion was removed and placed in cold Krebs–Ringer phosphate buffer, pH 7·4, with glucose (20 mmol). The period between operation and the onset of incubation did not exceed 30 min. For incubations 20–100 mg of amniotic epithelium was used. The tissue was placed into the incubation flask containing about 0·5 μCi of [4-^{14}C]-dehydroepiandrosterone dissolved in 0·05 ml of propylene glycol, and 3 ml of Krebs–Ringer phosphate buffer with glucose. The final concentration of the substrate in the incubation medium was about 3 μmol. As blanks 10 mg of freeze-dried human albumin was incubated with labeled steroid instead of tissue. No cofactors were added. The incubation in oxygen atmosphere at 37°C was continued for 60 min. The reaction was stopped by 6 ml of ethyl acetate and 20 μg of authentic 7α-hydroxydehydroepiandrosterone was added.

In separate experiments non-radioactive DHA (300 μg) was incubated with 100–200 mg amniotic epithelium in 3 ml of buffer. Five incubations, i.e. 700 mg of epithelium from the 9th to the 12th week of pregnancy, were worked up and the pooled reaction mixture was used for the identification of the non-radioactive metabolite.

Extraction and chromatography. Extraction of steroids was carried out with ethyl acetate (6 ml) and chloroform (3 ml) and the combined organic phases were evaporated *in vacuo*. Dry residues were chromatographed on Whatman paper No. 1 in the solvent system cyclohexane–toluene–methanol–water (9:1:8:2 by vol.) (system I). The polar zone (R_F 0·0–0·10) was cut out, steroids were eluted with methanol and re-chromatographed on paper in the solvent system toluene–methanol–water (2:1:1 by vol.) (Bush B4).

The chromatographic standard 7α-OH-DHA was visualized by antimony-III-chloride reagent, radioactive steroids were localized by autoradiography.

Incubation products of non-radioactive DHA with amniotic epithelium were chromatographed first on silica gel plates Silufol in the solvent system benzene–methanol, (3:1 v/v) (system III), and the zone of 7α- and 7β-OH-DHA was eluted with methanol and rechromatographed on paper in solvent system Bush B4.

Measurement of radioactivity. The radioactivity corresponding to [4-^{14}C]-DHA and [4-^{14}C]-7-OH-DHA was measured in an aliquot part of methanolic eluates of spots from paper chromatograms. The measurements were carried out in a liquid scintillation spectrometer Betaszint BF 5000 Berthold–Frieseke in 10 ml of scintillation fluid SLT 4l.

Methods for identification of 7-OH-DHA. For the identification of radioactive hydroxymetabolites the residual material of 8 chromatographic spots remaining after the measurement of radioactivity was combined. An amount corresponding to 639,000 d.p.m. of labeled metabolite was worked up for identification. Characterization of the metabolite was based on its chromatographic mobilities and of those of its oxidation and reduction products. Oxidation was carried out with 2,3-dimethyl-5,6-dicyano-1,4-benzoquinone, a specific oxidant of allylic alcohols [6]. Methanolic sodium borohydride was used as reducing agent.

For additional chromatographic characterization separation in the following solvent systems was used: system II = heptane–toluene–methanol–water, (9:11:16:4 by vol.), development 48 h, Bush B5 solvent system = benzene–methanol–water, (2:1:1 by vol.) and system IV = tetrachloromethane on triethylene glycol soaked paper (30% triethylene glycol in methanol), development 24 h.

Gas chromatography–mass spectrometry. The combined incubation products of non-radioactive DHA with amniotic epithelium after thin-layer and paper chromatography were subjected to GC–MS as trimethyl silyl ethers using 5α-cholestane (retention time 489 s) as internal standard on a 1·5% SE-30-Chromosorb WHP column, column temperature 240°C. An LKB 2091 GC–MS was employed. The mass spectra were recorded on magnetic tape and treated in a PDP 11/10 computer.

RESULTS

After incubation of [4-^{14}C]-DHA with amniotic epithelium the metabolic turnover of the steroid was found to be surprisingly high. In the first trimester of pregnancy approx. 15–36%, in the second trimester even 78% of incubated DHA was transformed to its metabolites. The overall yield of radioactivity extracted from the incubation mixture, i.e. the recovered substrate and the metabolites, amounted to 92·1 ± 7·0% of the radioactivity incubated.

Besides the reported conversion of [4-^{14}C]-DHA to [4-^{14}C]-5-androstene-3β-17β-diol [4] and to 4-ene-3-ketones (which will be described elsewhere), an appreciable amount of radioactivity was recovered in material which by its chromatographic mobility corresponded to monohydroxylated DHA and which was identified as follows.

Compound 1. The chromatographic mobilities of labeled metabolite were identical with those of authentic 7α-OH-DHA on paper in solvent system Bush B4 (R_f 0·20), B5 (R_f 0·39), in solvent system II (0·30 cm./h) and IV on triethylene glycol soaked paper (0·104 cm./h) and on thin-layer of silica gel in solvent system III (R_f 0·48). Selective oxidation of allylic alcoholic function yielded a material identical in chromatographic mobility with 3β-hydroxy-5-androstene-7,17-dione (solvent system IV, 0·458 cm./h). The main product of borohydride reduction was chromatographically identical with 5-androstene-3β,7α,17β-triol (Bush B5, R_f 0·10).

After incubation and rechromatography (solvent system III and Bush B4) of metabolite arising from non-labeled DHA the material with chromatographic mobility of 7α-OH-DHA, giving a blue colour with antimony-III-chloride reagent, was subjected to gas chromatography–mass spectrometry as the trimethyl silyl ether derivative. The peak with relative retention time t_R 0·57, identical with 7α-OH-DHA, gave a mass spectrum with a molecular ion at *m/e* 448 and a very prominent base peak at *m/e* 358 (M-90). Peaks were also seen at *m/e* 433 (M-15), 343 (M-(90 + 15)), 253 (M-(2 × 90 + 15)) 208 and 129 and the spectrum was identical to that of authentic 7α-OH-DHA and to that published by Jänne and Vihko[7].

The data show that compound 1 is 7α-hydroxydehydroepiandrosterone.

Compound 2. The chromatographic mobilities of 4-^{14}C compound 2 were identical to those of 7β-OH-DHA in solvent system II (0·27 cm./h) and in Bush B4 (R_f 0·28).

The trimethyl silyl ether derivative of compound 2 after incubation of non-labeled substrate had a relative retention time t_R = 0·72 identical to that of 7β-OH-DHA and a mass spectrum with a molecular ion at *m/e* 448 and peaks identical to those of the mass spectrum of reference 7β-OH-DHA and very similar to those of the mass spectrum of 7α-OH-DHA. In the light of these results, compound 5 was identified as 7β-hydroxydehydroepiandrosterone.

The ratio of the 7α- to the 7β-epimer varied according to the substrate concentration and was 1:40–1:50 when incubating [4-^{14}C]-DHA and 1:4 when using non-labeled DHA.

Compound 3: Radioactive material found on paper chromatograms developed in Bush B4 solvent system in the area of 3β-16α-dihydroxy-5-androsten-17-one (R_F 0·56) amounted to only 1/400 of radioactivity of 7α-OH-DHA (in incubation mixture with tissue from the 12–13th week of pregnancy) or 1/40–1/70 of 7α-OH-DHA radioactivity in experiments with

Table 1. The conversion of [4-^{14}C]-DHA (1·10–1·27.10^6 dpm) to [4-^{14}C]-7α-OH-DHA in human amniotic epithelium

Foetal age weeks'	Sex	Weight of tissue incubated mg	No. of parallels n	Amount of DHA metabolized %[a]	Amount of 7α-OH-DHA formed dpm[b]	Amount of 7α-OH-DHA formed %[a]
7	?	45	1	15.3	6 696	0.54
8 - 9	?	100	1	35.0	1 848	0.17
8 - 9	?	81	1	55.0	8 527	0.78
9 -10	male	70	1	20.7	12 747	1.02
12	female	100	1	27.6	25 643	2.06
12	?	20	2[c]	20.9	54 035	4.27
12 - 13	male	100	2[c]	36.0	117 380	9.74
21 - 22	female	100	4[c]	68.9	421 787	33.84
21 - 22	male	100	2[c]	78.3	448 112	35.96
22	male	100	1	66.8	334 669	26.85
22 - 23	male	100	1	65.2	431 176	34.60

[a] relative to [4-^{14}C]-DHA incubated as substrate

[b] calculated per 100 mg of tissue incubated

[c] yields given as mean values of parallel experiments

amniotic epithelium from the 21–23rd week of gestation. No attempt for further identification of 16α-hydroxylated derivative of DHA was done for the apparent relative lack of 16α-hydroxylase in amniotic epithelium.

The relation of 7-hydroxylation to foetal age. The yields of [4-^{14}C]-7α-hydroxydehydroepiandrosterone in d.p.m. and relative to radioactivity incubated are shown in Table 1. The amount of the substrate converted to 7α-hydroxylated metabolites ranged from 0·2 to 36·0%. It is evident that 7α-hydroxylation of DHA occurs *in vitro* in amniotic epithelium as early as in the 7th week of gestation and that the yields of 7α-hydroxylated metabolites increase with progressing pregnancy. In midpregnancy, 7α-hydroxydehydroepiandrosterone becomes the major metabolic product of dehydroepiandrosterone in human amniotic epithelium under conditions used in present experiments.

DISCUSSION

The ability to hydroxylate DHA to 7α and 7β-hydroxydehydroepiandrosterone has been demonstrated in various human and animal tissues. 7-Hydroxylation was found to occur *in vitro* in many human foetal tissues including liver, adrenals, testis, skin, muscle, thymus, spleen, heart, kidneys, lung, gut and chorion [8, 9]. The onset of the 7-hydroxylating activity varied from 8th week of gestation (liver and chorion) to 16th week (adrenals). In the present study the presence of the mixed-function oxidase systems which hydroxylate DHA in positions 7α- and 7β- has been demonstrated in human amniotic epithelium. The 7α-hydroxylation prevails over the 7β-hydroxylation, similarly as in the foetal liver [9]. In the incubations of DHA with chorion 7β-epimer was the more abundant metabolite [9].

7-Hydroxylating rates progressively increase in amniotic epithelium from foetuses of both sexes throughout the foetal development from 7th to 23rd week of gestation. No sexual dependence could be seen in the enzyme activity.

Although 16α-hydroxylated C_{18}- and C_{19}-steroids are normal and quantitatively important constituents of amniotic fluid [2, 10], 16α-hydroxylation of DHA, if any, was far less intensive than 7α-hydroxylation in human amniotic epithelium. In spite of many similar features in both mixed-function oxidases there is an apparent difference between 7α- and 16α-hydroxylase in the pattern of their age and gonad dependence in the rat liver [11]. The activity of 16α-hydroxylase as compared with 7α-hydroxylase in human amniotic epithelium is so low that this tissue can be neglected as quantitatively important source contributing to the content of 16α-hydroxydehydroepiandrosterone in amniotic fluid. However, in respect of high 7-hydroxylating activity especially in the second trimester of pregnancy, the human amniotic epithelium must be considered as an actively steroid metabolizing tissue.

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