3 β -Hydroxysteroid dehydrogenase activity and gestagen concentrations in bovine cotyledons and caruncles during gestation and parturition

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3 β -Hydroxysteroid dehydrogenase (3 β -HSD) activity in bovine cotyledons was much higher than in caruncles throughout the gestation period. The activity of this enzyme in cotyledons increased greatly to 150.6 ± 5.8 pmol min⁻¹ mg⁻¹ protein during the seventh month of gestation, reached a peak of 221.0 ± 34.9 pmol min⁻¹ mg⁻¹ protein during the eighth month, and decreased at parturition. Progesterone and 20 α -hydroxyprogesterone concentrations in cotyledons also increased sharply to 2.69 ± 0.30 and 2.15 ± 0.42 ng mg⁻¹ protein, respectively, during the seventh month of gestation, reaching peaks of 2.86 ± 0.47 and 2.51 ± 0.36 ng mg⁻¹ protein, respectively, during the eighth month and decreasing at parturition, in a manner similar to the activity of 3 β -hydroxysteroid dehydrogenase. The fluctuation of 17 α -hydroxyprogesterone concentration in cotyledons was different from that of progesterone and 20 α -hydroxyprogesterone. These findings indicate that the activity of 3 β -hydroxysteroid dehydrogenase in the placenta is enhanced during the third trimester, and progesterone synthesized in the cotyledons is converted concurrently to 20 α -hydroxyprogesterone before progesterone is transferred to the fetal blood.

Introduction

It has been reported that progesterone is not produced in the bovine placenta, as bovine uterine arteriovenous progesterone concentrations do not alter in this tissue (Comline *et al.*, 1974; Ferrell *et al.*, 1983). However, many other studies (Ainsworth and Ryan, 1967; Wiener, 1976; Inaba *et al.*, 1983; Reimers *et al.*, 1985; Conley and Ford, 1987) have demonstrated and confirmed progesterone synthesis in tissue cultures of placentae. In contrast to the attenuation in the function of bovine corpora lutea during the second half of gestation (Erb *et al.*, 1968; Shemesh *et al.*, 1983), progesterone concentration in the blood has been shown to increase substantially near term (Short, 1958; Stabenfeldt *et al.*, 1970). Such an increase in the progesterone concentration during late gestation has been attributed to production by the placenta (Gomes and Erb, 1965; Stabenfeldt *et al.*, 1970) and the adrenals (Wendrof *et al.*, 1983). However, our knowledge of gestagen synthesis in the bovine placenta is limited. The aim of this study was to investigate progesterone synthesis in both cotyledons and caruncles by measuring the synthesis-related enzyme, 3 β -hydroxysteroid dehydrogenase (3 β -HSD), and gestagen concentrations in the placenta with respect to the stage of gestation.

Materials and Methods

Animals and treatments

Cotyledons and caruncles were isolated from 32 Holstein cows (28 gestations and four normal parturitions). The number of cows examined during gestation months 4–9 and at parturition was 4, 4, 4, 6, 5, 5 and 4, respectively. The cotyledons and caruncles were separated manually within 30 min of slaughter or parturition. These isolated placentae were washed with cold physiological saline and were frozen immediately with dry ice and stored at -80°C until assayed. The stage of gestation was estimated by determining fetal crown–rump length (Arthur *et al.*, 1982).

Assay of 3 β -HSD activity

Determination of enzymatic activity was based on the substrate metabolism methods of Seki *et al.* (1987) and Hirato *et al.* (1982). [¹⁴C]Pregnenolone (specific activity, 57.2 Ci mmol⁻¹) was obtained from New England Nuclear Co. (Boston, USA). NAD was purchased from Oriental Koubo (Tokyo). Cotyledons and caruncles were homogenized in nine volumes of 0.25 mol sucrose–phosphate buffer l⁻¹ in a Teflon–glass homogenizer (Braun Melsungen, Wendelsteinstr.,

Germany) at 1000 r.p.m. for 45 s. Enzyme preparations (microsomal fraction) were obtained after centrifugation at 10 000 *g* for 15 min at 4°C from the supernatant, which was then re-centrifuged at 105 000 *g* for 1 h at 4°C. The reaction medium contained 60 µl microsomal fraction, 740 µl 5 mmol MgCl₂ l⁻¹ in 0.25 mol sucrose-phosphate buffer l⁻¹ and 100 µl 0.8 µmol NAD l⁻¹, and 100 µl [¹⁴C]pregnenolone (0.3 µCi). After aeration with CO₂ for 2 min, the reaction was allowed to continue for a further 10 min at 37°C under constant agitation (120 r.p.m.), and was stopped by adding 1 ml of 1 mol HCl l⁻¹. Non-reacted substrate and metabolites were removed with two rinses of diethyl ether and dried under a stream of N₂.

Pregnenolone was separated from progesterone using silica gel G thin-layer chromatography, with a development solvent of benzene:ethyl acetate (2:1). The scanner confirmed the site of radioactivity on the thin-layer chromatography; progesterone was removed by scraping the resin at this site, followed by hormonal extraction carried out twice with diethyl ether. The radioactivity of the extract was measured with a liquid scintillation counter (Beckman Instruments, CA). As the present assay induced the synthesis of [¹⁴C]progesterone only from [¹⁴C]pregnenolone, the quantity of [¹⁴C]progesterone produced served as an index of 3β-HSD activity. Enzyme activity was calculated from the percentage of product formed relative to the total radioactive steroid recovered. Recovery after extraction and chromatography was 61.4 ± 6.2% (*n* = 10). The intra-assay and interassay coefficients of variation in this 3β-HSD activity assay were 8.2% and 13.7% (*n* = 10), respectively. The protein concentration in the sample was determined by the method of Lowry (1951), using BSA as the reference protein.

Determination of progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone concentrations

Progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone concentrations in cotyledons and caruncles were determined by the radioimmunoassay methods of Makino (1973) and Tanemori (1978), with slight modifications. Labelled hormones, [1,2-³H]progesterone, [1,2,6,7-³H]20α-hydroxyprogesterone, and [1,2,6,7-³H]17α-hydroxyprogesterone, were purchased from Amersham International (Amersham, Bucks). Antisera against progesterone-3-carboxy methyl oxime-BSA (CMO-BSA), 20α-hydroxyprogesterone-CMO-BSA, and 17α-hydroxyprogesterone-3-CMO-BSA were supplied by Teikoku Hormone Mfg Co. (Tokyo). Crossreactivities of the progesterone antiserum were 62.2% for 5α-pregnanedione and 6.3% for pregnenolone; the crossreactivity of the 20α-hydroxyprogesterone antiserum was 5.4% for 20β-hydroxyprogesterone, while the crossreactivities of 17α-hydroxyprogesterone antisera were 7.9% for progesterone and 3.2% for 20α-hydroxyprogesterone, respectively.

The gestagen concentrations were determined by subjecting the dried residue of the diethyl ether extract of the homogenized sample (1–50 µl) before ultracentrifugation to liquid chromatography elution with a Sephadex LH-20 column, using a mobile phase of hexane:benzene:methanol (82.5:10:7.5, v:v:v). The rates of recovery after extraction and chromatography were 92.2 ± 4.2% for progesterone, 82.5 ± 4.0%

for 20α-hydroxyprogesterone and 75.1 ± 3.8% for 17α-hydroxyprogesterone (*n* = 15). The lower limit of accurate quantitation was 10 pg less than that specified in the respective assay procedures for progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone. The intra-assay and inter-assay (*n* = 15) coefficients of variation for progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone were 8.2%, 9.8% and 9.2%, and 12.3%, 14.8% and 17.4%, respectively. In determining the interassay and intra-assay coefficients of variation, the concentrations of progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone in the control samples (*n* = 15) were 1.54 ± 0.40, 1.05 ± 0.30, and 0.51 ± 0.16 ng mg⁻¹ protein, respectively.

Statistical analysis

Statistical significance was verified by Duncan's multiple range test and by determining the correlation coefficients for the specific effects of site, month of gestation, and any interaction between these and the concentrations of 3β-HSD, progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone.

Results

Profile of the method for determining 3β-HSD activity

To decide on the sample quantity and the incubation time used, we evaluated the microsomal fraction from the cotyledon tissue during the eighth month of gestation; a linear relationship was obtained with up to 0.55 mg protein and 20 min (Fig. 1a, b). We therefore used protein samples of about 0.2 mg and an incubation time of 10 min in this study. The Michaelis constant (*K_m*) and the maximum 3β-HSD reaction velocity (*V_{max}*) for cotyledons during gestation month eight were derived from a Lineweaver-Burk plot of enzyme activity against substrate concentration. The *K_m* and *V_{max}* values in the cotyledons were 100 nmol l⁻¹ and 100 pmol min⁻¹ mg⁻¹ protein, respectively (Fig. 1c).

3β-HSD activity in cotyledons and caruncles

The 3β-HSD activity in the cotyledons increased significantly (*P* < 0.05) between gestation months 4 and 7, reached a peak at gestation month 8, and decreased immediately after parturition. The activity of 3β-HSD was low in the caruncles throughout the experimental period, and was significantly (*P* < 0.01) lower than in the cotyledons (Fig. 2a).

Progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone concentrations in cotyledons and caruncles

The concentrations of progesterone and 20α-hydroxyprogesterone in cotyledons are shown (Fig. 2b, c). The coefficients of correlation (*r*) between 3β-HSD activity and progesterone, 3β-HSD activity and 20α-hydroxyprogesterone,

Fig. 1. Characterization of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. (a) Relationship to protein content in the bovine cotyledon during the eighth month of gestation. (b) Relationship to incubation time, using 0.2 mg of the bovine cotyledon during the eighth month of gestation. (c) Lineweaver-Burk plot showing the relationship between bovine placental (microsomal fraction) 3β-HSD activity (1/V) and [¹⁴C]pregnenolone concentration (1/S × 100).

and progesterone and 20α-hydroxyprogesterone in cotyledons were 0.811, 0.721 and 0.872 ($P < 0.01$), respectively. Cotyledons expressed significantly ($P < 0.01$ or $P < 0.05$) higher concentrations of progesterone and 20α-hydroxyprogesterone than did caruncles throughout the gestation period.

Fig. 2. (a) Activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) and concentrations of (b) progesterone, (c) 20α-hydroxyprogesterone, and (d) 17α-hydroxyprogesterone in bovine cotyledons (■) and caruncles (□) during gestation. Histograms show means ± SEM. Asterisks indicate significant differences compared with values during month 4 of gestation: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The concentration of 17α-hydroxyprogesterone in cotyledons at gestation months 4 and 5 was significantly ($P < 0.01$ or 0.05) higher than that of 20α-hydroxyprogesterone (Fig. 2d); however, from gestation month 7–9, the concentration of 17α-hydroxyprogesterone was significantly ($P < 0.01$ or 0.05) lower than that of progesterone and 20α-hydroxyprogesterone.

In caruncles, progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone concentrations showed no apparent changes throughout the gestation period.

Discussion

Documentation of progesterone synthesis in relation to the progress of gestation is limited. Inaba *et al.* (1983) demonstrated increases in progesterone synthesis from gestation months 7.5–9 in cows; however, only one case per month was sampled. Their finding is similar to the pattern of 3β -HSD activity in the study reported here. Moreover, in this study, changes in progesterone concentrations in cotyledons were very similar to the fluctuations in 3β -HSD activity. The incidence of miscarriages in pregnant cows luteoectomized after gestation day 200 is low (Estergreen *et al.*, 1967). Our findings have indicated that this phenomenon is probably due to the functional role of progesterone released from the placenta rather than from the maternal adrenals.

We found that 3β -HSD activity was higher in the bovine cotyledons than in the caruncular placentae. This finding is in agreement with findings that the fetal cotyledon is the primary site of placental progesterone (Conley and Ford, 1987; Gross and Williams, 1988) and oestrogen synthesis (Hoffmann *et al.*, 1976; Tsumagari *et al.*, 1993). We also found that progesterone concentration in caruncles is much lower than that in the cotyledons. Thus, the distribution of progesterone in cotyledons and caruncles appears to differ from that of oestrogen, as there is no significant difference between the concentration of oestrogen in cotyledons and caruncles (Tsumagari *et al.*, 1993), and two-thirds of the samples used in the previous study were also investigated in the study reported here. This difference in the distribution of the two steroids is surprising as the diffusion of steroids should be equal in maternal and fetal directions. In this study, we found that the concentration and fluctuation of 20α -hydroxyprogesterone in the cotyledons throughout the gestation period was very similar to that of progesterone. The ovine fetal placenta has been shown to convert progesterone to 20α -hydroxyprogesterone (Anderson *et al.*, 1975); furthermore, fetal erythrocytes in both sheep and cows have been shown to contain abundant quantities of the enzyme responsible for converting progesterone to 20α -hydroxyprogesterone (Nancarrow, 1983). The conversion of progesterone to 20α -hydroxyprogesterone is reported to be a reversible reaction (Nancarrow and Seamark, 1968). Our current and some previous findings (Nancarrow and Seamark, 1968; Anderson *et al.*, 1975; Nancarrow, 1983) indicate that the progesterone produced in the fetal cotyledon is rapidly converted to 20α -hydroxyprogesterone before diffusing to the maternal caruncle, which explains why the maternal site has a lower progesterone concentration than does the fetal site. The progesterone concentration in bovine fetal plasma is very low (Challis *et al.*, 1974; Hoffmann *et al.*, 1976). These findings also support the idea that the conversion of progesterone to 20α -hydroxyprogesterone occurs in the fetal compartment. A low progesterone concentration in the fetus is beneficial for its development and survival.

Although we found that cotyledons had a relatively high concentration of 17α -hydroxyprogesterone during the fourth month of gestation, there were no subsequent changes in the concentration of this hormone accompanying the progress of fetal development. Moreover, there was no apparent correlation between the concentrations

of 17α -hydroxyprogesterone and progesterone, implying that 17α -hydroxyprogesterone was probably derived from 17α -hydroxypregnenolone.

Our findings here indicate that increases in placental 3β -HSD activity persist from gestation month 7–9, as progesterone synthesized in the cotyledon is converted concurrently to 20α -hydroxyprogesterone before being transferred to the fetal blood during this period.

The authors wish to thank V. L. Estergreen for reading the manuscript. This study was supported, in part, by a Grant-in-Aid for Scientific Research (B) 62480090 from the Ministry of Education, Science and Culture of Japan.

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