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ESTRADIOL-15α-HYDROXYLATION: A NEW AVENUE OF ESTROGEN METABOLISM IN PERI-IMPLANTATION PIG BLASTOCYSTS

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Summary—Pig blastocysts have the capacity to convert estradiol into catechol estrogens. Our present study shows that they also have the capacity to hydroxylate estradiol in cycloaliphatic C-atom 15, and this aliphatic hydroxylation reaction is more predominate than the aromatic hydroxylations. The conversion of [4-^{14}C]estradiol to [4-^{14}C]15α-hydroxyestradiol by mitochondrial-rich/microsomal fractions was examined by isolation of this product using reversed phase high-performance liquid chromatography (HPLC) attached to a radiometric flow detector, and its identification by gas chromatography-mass spectrometry. The enzyme kinetics for estrogen 15α-hydroxylase were performed in the pig blastocyst obtained on Day 13 of pregnancy (Day 0 = first acceptance of the male). The enzyme follows classical Michaelis–Menten kinetics. The apparent K_ms for estradiol were 2.47 and 1.85 μM, and the apparent V_{max}s were 0.25 and 0.197 nmol/mg/min in the mitochondrial-rich and microsomal fractions, respectively.

The enzyme activity was inhibited by different steroidal compounds and non-steroidal estrogens, as well as by CO, SKF-525A, piperonyl butoxide and antibody to cytochrome *P*450 reductase.

Ontogenesis of the blastocyst's estrogen 15α-hydroxylase follows a similar pattern to that of estrogen-2/4-hydroxylase. Thus, highest activity was observed on Days 12 and 13 and lowest was on Day 15 of pregnancy. Furthermore, the enzyme is abundant primarily in the extraembryonic tissues rather than in the embryo proper. The abundance of the enzyme in the extraembryonic tissues, and its surge at a critical time of pregnancy recognition and just prior to implantation suggest that 15α-hydroxylated estradiol could be involved in these processes.

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Abbreviations and Trivial Names: Aminoglutethimide, α-(*p*-aminophenyl)-α-ethyl glutarimide; androstenedione, 4-androstene-3,17-dione; cholesterol, 1,4,6-androstatien-3,17-dione; diethylstilbestrol, 3,4-bis-(*p*-hydroxyphenyl)-3-hexane; hexestrol, meso-3,4-bis[4-hydroxyphenyl]-hexane; 2-hydroxyestradiol (2-OH-E_2), 1,3,5(10)-estratrien-2,3,17β-triol; 4-hydroxyestradiol (4-OH-E_2). 1,3,5(10)-estratrien-3,4,17β-triol; 15α-hydroxyestradiol (15α-OH-E_2), 15α-hydroxy-1,3,5(10)-estratriene-3,17β-diol; α-naphtoflavone, 7,8-benzoflavone; estradiol (E_2), 1,3,5(10); estratriene-3,17β-diol; estriol (E_3), 1,3,5(10)-estratriene-3,16α,17β-triol; estetrol (E_4), 1,3,5(10) estratriene-3,15α,16α,17β-tetrol; estrone, 1,3,5(10)-estratrien-3-ol-17-one; piperonyl butoxide, 3,4-methylenedioxy-6-propylbenzyl butyl diethyleneglycol ether; pregnenolone, 3β-hydroxypren-5-en-20-one; progesterone, pregn-4-en-3,20-dione; SKF525-A, diethylaminoethyl-2,2-diphenyl valerate HCl; testosterone, 17β-hydroxy-4-androsten-3-one. *Enzymes*: NADPH-cytochrome reductase (EC 1.6.2.4); cytochrome-c-oxidase (EC 1.9.3.1); glucose-6-phosphatase (EC 3.1.3.9); steroid-16α-hydroxylase (EC 1.14.14.1).

INTRODUCTION

In the pig, maternal recognition of pregnancy is initiated on Days 10–12 of pregnancy and is attributed to signals arising from the blastocyst [1]. Although this time period coincides with the initiation of a number of events including estrogen synthesis by the blastocyst [2–4] leading to increases in protein, calcium and prostaglandins within the uterine lumen [5, 6], the issue of pregnancy recognition has not yet been settled. A surge of estrogen 2/4-hydroxylase (E-2/4-H) activity occurs in the pig blastocyst on Days 11–13 of pregnancy [7], and the importance of its reaction products (catechol estrogens) in pregnancy recognition has been suggested. In the previous study [7], the assay of E-2/4-H involved incubation of the blastocyst homogenate with radioactive estradiol in the presence of an NADPH-generating system, selective adsorption of the catechol estrogens on neutral alumina, separation by thin-layer chromatography, and quantitation by liquid scintillation counting. Recently, omission of the alumina chromatography, and replacement of the thin-layer chromatography by reversed phase high-

performance liquid chromatography (HPLC) led us to detect a new reaction product of estradiol-17β (E_2) produced by the pig blastocyst in an NADPH-driven reaction. This is, in fact, one of the major products formed and has been identified as 15α-hydroxy-estradiol-17β (15α-OH-E_2) by gas chromatography–mass spectrometry (GC–MS). To date, the existence of 15α-hydroxylation reaction has only been established in micro-organisms [8], bovine [9] and human [10] adrenals and in the human fetal liver [11–14], and more so, the enzyme has not been well characterized in any of these tissues.

In the present study, we have examined estradiol-15α-hydroxylase (E_2-15α-H) in peri-implantation blastocysts of pigs under initial velocity conditions using a radiotracer method. Our main objectives were to examine the kinetic parameters of this enzyme, determine the temporal relationship between this enzyme activity and the stage of blastocyst development on different days of pregnancy (Days 11–20), and to determine which tissues of the blastocyst exhibit this activity. We have also examined whether the formation of 15α-OH-E_2 is catalyzed by cytochrome *P*450 (cyt *P*450). Furthermore, in order to gain insights into the structural requirements of the active site, as well as the regulation of the enzyme by different steroids in the pig blastocyst, the effects of various steroidal and non-steroidal estrogens, haloestrogens, catecholestrogens, cholesterol, androgens and progestins on E_2-15α-H were also examined.

MATERIALS AND METHODS

Reagents

HPLC-grade methanol, water and sodium acetate were purchased from Fisher Scientific (St Louis, Mo.), and glacial acetic acid (HPLC-grade) was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Radioactive estradiols [6,7-^{3}H(N)E_2 (42.0 Ci/mmol) and [4-^{14}C]E_2 (56.4 mCi/mmol) were purchased from New England Nuclear (Boston, Mass). Two-hydroxy-estradiol (2-OH-E_2) and 4-hydroxy-estradiol (4-OH-E_2) were purchased from Steraloids (Wilton, N.H.). 2-Bromo-estradiol and 2-fluoro-estradiol were gifts from Drs R. W. Brueggeimer and J. Liehr respectively. 15α-OH-E_2 was a gift from Drs S. Sassa and A. Kappas. SKF-525A was obtained from Smith–Kline and French Laboratories (Philadelphia, Penn.) and piperonyl butoxide from Chemalog, Chemical Dynamics Corp. (South Plainfield, N.J.). Antibody to rat microsomal NADPH-cytochrome *P*450 reductase was prepared by Dr A. Parkinson as mentioned previously [15] and was provided by him. Antibody to estriol was obtained from Diagnostic Systems, Webster, Texas. All other reagents were purchased from Sigma Chemical Co. (St Louis, Mo.).

Animals and tissue preparation

Pigs (both sows and gilts) were checked daily for estrous and mated or artificially inseminated at first detection of estrous and again 24 h later. The onset of estrous was defined as Day 0 of pregnancy. Blastocysts were recovered by flushing the uterus with Dulbecco's phosphate buffered saline (PBS) on Days 11–20 of pregnancy. These were rinsed in PBS and immediately frozen and stored at −70°C. Blastocysts were collected once from each pig. A number of blastocysts on Days 13 and 20 were separated into embryonic and extraembryonic tissues.

Tissues were homogenized in 0.25 M sucrose in a glass tissue homogenizer with a Teflon pestle, and centrifuged at 1000 *g* for 15 min. The supernatant fluids, in most cases, were then centrifuged at 105,000 *g* for 60 min to obtain mixed mitochondrial–microsomal fractions. In some cases, the 1000 *g* supernatant fluids prepared from the blastocysts of Day 13 of pregnancy were centrifuged at 10,000 *g* for 20 min to obtain mitochondrial rich pellet (as evidenced by higher cytochrome C oxidase), and the 10,000 *g* supernatant fluid was again centrifuged at 105,000 *g* for 60 min to obtain microsomal pellet (as evidenced by higher glucose-6-phosphatase activity). All these procedures were carried out at 4°C. The pellets were rehomogenized gently with a minimum volume of 0.25 M sucrose, and were used for assay on the same day. The protein contents were measured by the Bradford method [16] using bovine serum albumin as a standard.

Incubations

General conditions for the assay were as follows: aliquots of the mitochondrial–microsomal homogenate (50 μl containing 100 μg protein) were incubated at 37°C for 30 min with 100 μl of reaction mixture containing 0.075 μCi [4-^{14}C]E_2-17β (56.5 mCi/mmol) substrate (10 μM), 10 mM ascorbic acid (to protect catecholestrogens from oxidation) and NADPH (1.5 mM) in Hepes–Tris (0.05 M:0.05 M) buffer at pH 7.5. Reactions were terminated by adding 100 μl of 1 M hydrochloric acid. Reaction tubes were cooled on ice and the reaction products and unreacted substrate were extracted with ethylacetate. Ethylacetate extracts were evaporated to dryness using a vortex evaporator, and redissolved in methanol for HPLC analysis. Blank values were obtained by using samples not containing NADPH.

High-performance liquid chromatography (HPLC)

Separation of the reaction products of E_2 was performed using an LC300 liquid chromatograph (Bioanalytical Systems, West Lafayette, Ind.) equipped with a flow detector (Flo-One Model IC Radiomatic, Tampa, Fla.). The flow detector measured radioactivity in a flowing system and is fully automated and microprocessor/computer controlled. The chromatograph is also equipped with an LC-4B amperometic detector (to detect catechol estrogens), LC-17 glassy carbon flow cell, Ag/AgCl reference RE-1 electrode, PM-30A dual piston pump, and

Rehodyne 7125 sample injector with a 20-μl injection loop. A 5-μM econosphere ODS column, 25 cm × 4.6 mm (Altech Assoc. Inc., Deerfield, Ill.) was used. The mobile phase was methanol/0.1 M sodium acetate/glacial acetic acid (50:42.5:7.5, v/v, pH 4.0) with a flow rate of 1 ml/min and back pressure of 2800 psi. Identifications of 2-OH-E_2 and E_2 were accomplished by comparing the retention times of the authentic samples (both radioactive and unlabeled). The major ^{14}C-peak, which could not be identified by our HPLC system was identified as 15α-OH-E_2 by gas chromatography–mass spectrometric (GC–MS) analysis comparing with the authentic sample. As this compound was found to be homogeneous and showed a single peak in gas chromatogram, the integrated peak area of this product in ^{14}C-channel of HPLC chromatogram was used for calculations.

To obtain enough unknown material for GC–MS analysis, several reactions were performed using unlabelled estradiol substrate and the fractions which corresponded with the peak area of the unidentified peak in the ^{14}C-channel were pooled. These pooled fractions were extracted 3 times with equal volumes of ethyl ether, that was removed by using vacuum evaporation at 40°C and the residue subjected to GC–MS analysis.

Gas chromatography–mass spectrometry (GC–MS)

Silylation was accomplished by heating the solvent-free steroid with *N,O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA, 20–50 μl) in a small glass vial with a reflon-lined cap for 3 h at 90°C. This reaction mixture was cooled and diluted with *n*-hexane (100–250 μl) and the resulting solution was used directly for GC–MS analysis.

Mass spectra were obtained using a Nermag R-10-10 quadruple mass spectrometer with 70 V electron energy and a 200°C ion source temperature. This mass spectrometer is connected to a Girdel model 31 gas chromatograph and the data are collected using a Nermag Spectral 30 PC based data system. The gas chromatograph was operated at 240°C using a falling rod injector. A capillary DB-5 column was used (30 m in length, 0.25 mm i.d.) with 1.5 bar helium pressure. The injector temperature was 220°C and the temperature of the interface to the mass spectrometer was 240°C.

RESULTS

Identification of the reaction products

Figure 1 shows a representative HPLC-chromatogram of the [^{14}C]-E_2 and its products formed as a result of the reaction with the mitochondrial–microsomal fraction of the pig blastocyst (Day 13) in the presence of NADPH. Three main peaks are observed (Fig. 1A). Peaks 2 and 3 have retention times of 10.6 and 15.2 min corresponding to 2-OH-E_2 and E_2 respectively. Peak 1 has a retention time of 4 min which is 0.7 min less than that of estriol (16α-hydroxy-estradiol). Inclusion of the alumina chromatography before HPLC shows only peak 2 (i.e. 2-OH-E_2) in the chromatogram (Fig. 1B), suggesting that product No. 1 does not have an OH group in the 2 or 4 position. This product cross-reacted extensively with the antibody to estriol, but attempts to recrystallize this ^{14}C-labeled product with estriol were futile. The antibody did not have any cross reactivity with epiestriol, estradiol or catecholestradiols.

Blastocyst homogenates (Day 13) incubated with 6,7-[^{3}H](N)E_2-17β and NADPH produced a negligible amount of radioactivity in the aqueous fraction. This result suggests that hydroxylation at the carbon 6 or 7 position did not occur. The remaining possible hydroxylation sites are 11, 14, 15 and 18.

In gas chromatography, the relative retention times (R_t) of the trimethyl silyl ether (TMS) derivatives of authentic 15α-hydroxyestradiol and product No. 1 isolated from HPLC were essentially equal [R_t of 15α-hydroxyestradiol $(TMS)_3$ = 27 min 46 s, R_t of product No. 1 $(TMS)_3$ = 27 min 42 s].

The mass spectra obtained with the TMS derivatives of the authentic 15α-OH-E_2 and product No. 1 are identical as shown in Fig. 2. Base peaks in both the spectra are the same (*m/e* 217). The other ions which are common in both the spectra are at: *m/e* 147, 156, 169, 182, 232, 245, 270, 387, 399, 489 and 504.

Kinetic properties of E_2-15α-H in pig blastocyst

The changes in E_2-15α-H activity as a function of time are shown in Fig. 3. For all subsequent studies the reaction time was 30 min. Essentially a zero intercept was obtained by stopping the reaction with 1N HCl at 0 h. Product formation was linear with increasing protein concentrations at least up to 100 μg in a reaction volume of 150 μl (Fig. 4). The amount of the product formed at 37°C was about 26% more than at 30°C. The enzyme activity was very low at pH 6.0 but increased with increasing pH up to 7.5; it decreased with higher pH (Fig. 5). However, the optimum pH of E-2-H of pig blastocyst was 8.0 using the same assay conditions. Formation of 15α-OH-E_2 was positively correlated with increasing concentrations of NADPH in the incubation medium (Fig. 6). However, even in the absence of any cofactor considerable amounts of the product was formed, which could be due to the presence of endogenous reduced nicotinamide. Because of this, incubations without added cofactors were used as blanks rather than "boiled tissue blank" in all assays. The apparent K_m for NADPH was determined using various NADPH concentrations and found to be 0.586 mM (Fig. 6). The K_m for NADH was about three times more than that of NADPH, suggesting the preference of the reaction for NADPH over NADH as cofactor.

After the initial velocity conditions were estab-

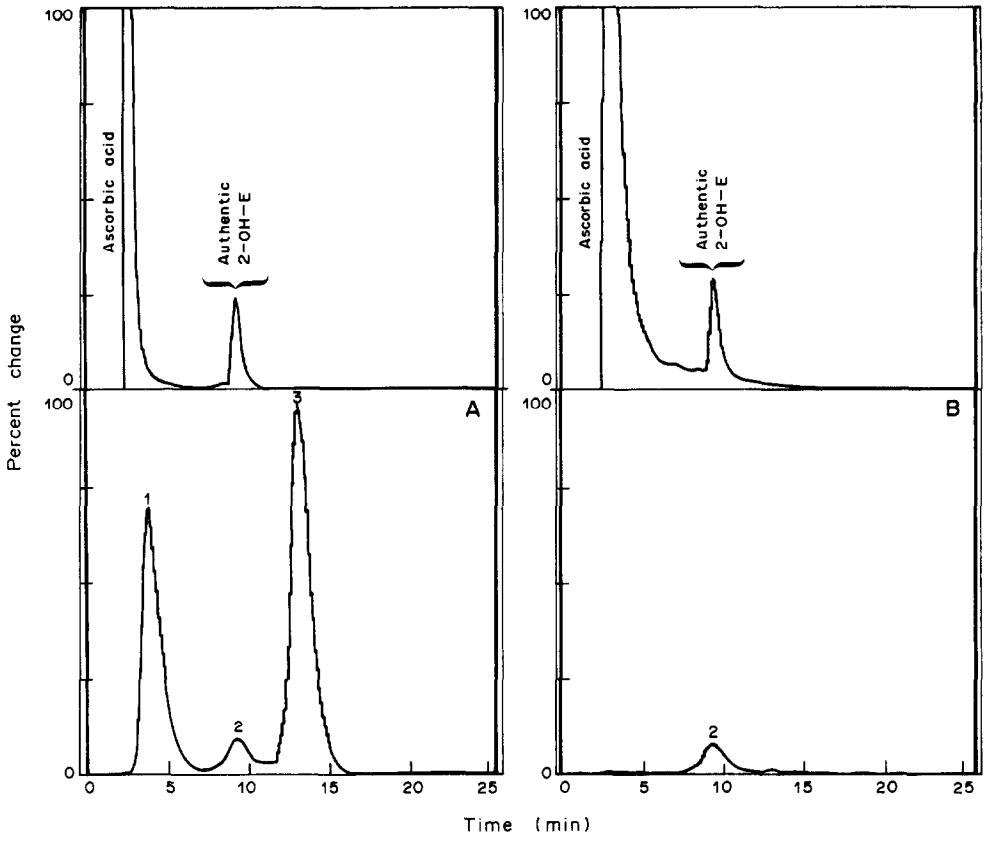

Fig. 1. HPLC Chromatograms (left tracings—radiometric; right tracings—electrochemical i.e. EC Channel) of the products obtained by reacting the mitochondrial–microsomal fraction of pig blastocyst (Day 13 of pregnancy) with [^{14}C]estradiol in the presence of NADPH. Column: ODS–Econosphere (4.6 × 250 mm). Mobile phase; methanol—0.1 M sodium acetate in water—glacial acetic acid, 50:42.5:7.5, 1 ml/min. The first peak in the EC channel corresponds to that of ascorbic acid used to prevent oxidation of catechol estrogen. (A) HPLC chromatogram of the reaction products and unreacted substrate before alumina chromatography. (B) same as A, but after alumina chromatography. Peak 1 in the radiometric channel could not be identified by this HPLC system, peak 2 corresponds to the authentic 2-hydroxy-estradiol in the EC channel, peak 3 is [^{14}C]estradiol.

lished, substrate dependence of the reaction was studied. As the enzyme activity was observed both in mitochondrial-rich and microsomal fractions, the substrate dependence was studied in both fractions from blastocysts (Day 13 of pregnancy). The data from the ^{14}C-labeled substrate assay of the change in velocity expressed in nmols of 15α-OH-E_2 formed per mg protein per min versus the substrate concentrations were plotted on double reciprocal plots. These yielded linear Lineweaver–Burk plots in both mitochondrial-rich (Fig. 7) as well as microsomal fractions (Fig. 8). Analysis of the data by computerized least squares regression gave an apparent K_m for E_2 of 2.47 μM and V_{max} of 0.25 nmol/mg protein/min for the mitochondrial-rich fraction, and a K_m of 1.85 μM and V_{max} of 0.197 nmol/mg protein/min for the microsomal fraction.

Effects of inhibitors of cytochrome P450 on E_2-15α-H activity

To determine whether 15α-OH-E_2 formation in pig blastocysts was mediated by cytochrome *P*450, various inhibitors of *P*450 including carbon monoxide (CO), α-naphthoflavone, piperonyl butoxide, SKF-525A and antibody to NADPH cytochrome *P*450 reductase were tested on E_2-15α-H activity. A mixture 95% CO + 5% O_2 caused 65% inhibition of the enzyme activity. Piperonyl butoxide (650 μM) inhibited the activity to 33%, whereas α-naphthoflavone (650 μM) was totally ineffective in inhibiting the enzyme activity. 15α-OH-E_2 production was inhibited in a dose-dependent manner by SKF-525A, a non-competitive inhibitor of *P*450, and by an antibody to NADPH cytochrome *P*450 reductase (Table 1). Aminoglutethimide, a potent inhibitor of *P*450-mediated cholesterol side chain cleavage and aromatase had essentially no effect of E_2-15α-H activity.

Effects of steroidal and non-steroidal compounds on E_2-15α-H activity

Effects of various estrogenic compounds and some other steroidal compounds on blastocyst E_2-15α-H activity *in vitro* were studied because these

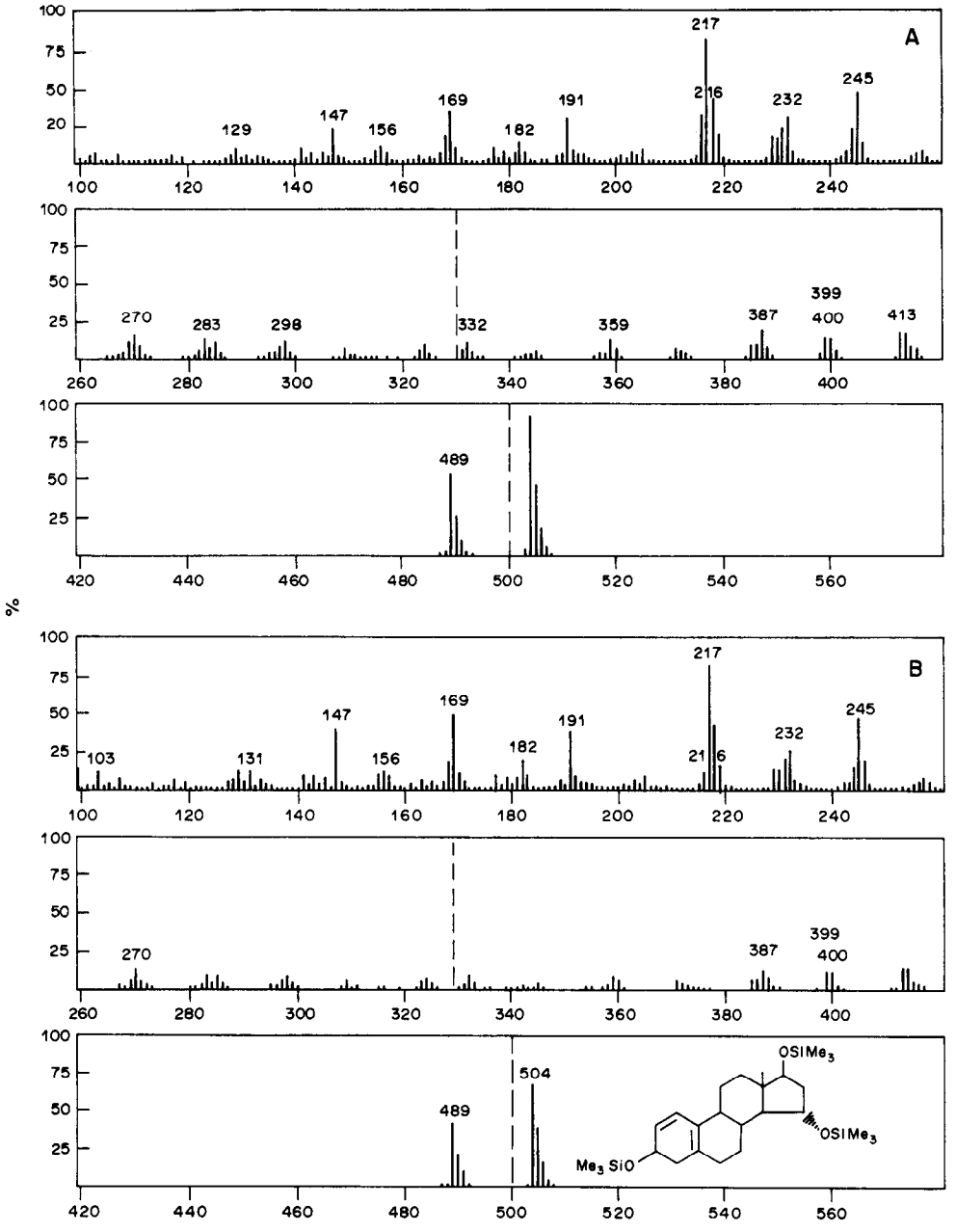

Fig. 2. Mass spectra of the reference 15-OH-estradiol-TMSi (A) and the TMSi derivative (B) of the compound (Peak 1 of Fig. 1A) produced by the pig blastocyst (Day 13).

agents might be useful for studing the kinetics of the enzyme and in the elucidation of the structural requirements of the active site. As shown in Table 2, all the compounds tested, except cholesterol and 4-hydroxy-androstenedione, had considerable inhibitory influence on the formation of 15α-OH-E_2 in a dose-dependent manner. Although, cholesterol did not inhibit, its side chain cleaved products, pregnenolone and progesterone were very strong inhibitors of E_2-15α-H. Both the phenolic estrogens, estrone and estriol were almost equally active in inhibiting enzyme activity. 2-Methoxy- and 2-hydroxy-estradiols were much more potent inhibitors than 4-hydroxy-estradiol. Androstenedione and testosterone, the immediate precursors of estrogens, two haloestrogens (2-bromo and 2-fluoroestradiols), which are very good inhibitors of catecholestrogen formation, and two non-steroidal estrogens, diethylstilbestrol and hexestrol, were also inhibitory to this enzyme.

Ontogenesis of E_2-15α-H

Figure 9 represents the ontogenesis of E_2-15α-H in pig blastocysts from Day 11 through Day 20 of pregnancy. On Day 11, the activity was about

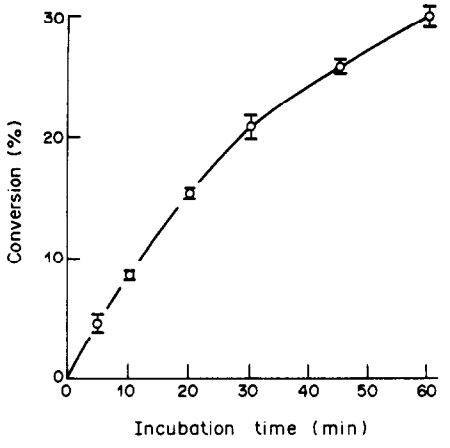

Fig. 3. Estradiol 15α-hydroxylase activity as a function of time. Aliquots of mitochondrial–microsomal fraction of pig blastocyst (Day 13) were incubated with [^{14}C]estradiol (10 μM) and NADPH (1.5 mM) as described under "Materials and Methods". The percentage of conversion of estradiol to 15α-hydroxy-estradiol was determined at various time intervals. Each point represents the average of three determinations (Mean ± SEM).

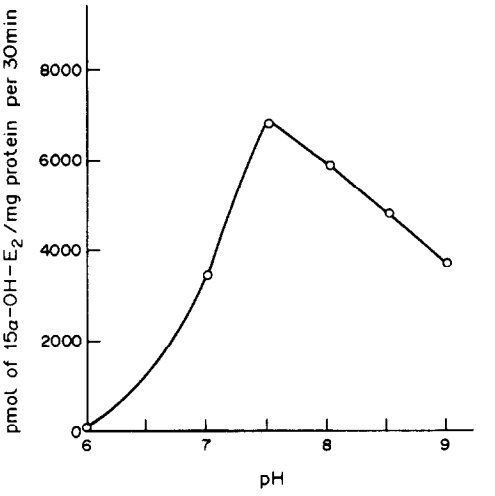

Fig. 5. The effect of pH on the estradiol-15α-hydroxylase activity obtained with 100 μg of blastocyst mitochondrial–microsomal protein. The pHs indicated were determined in mixtures of tissue, buffer and reaction mixture at 30°C. Each point represents average of two determinations. All other assays were performed at pH 7.5.

375 pmol/mg/30 min and this increased to a maximum on Days 12 and 13 (15–20-fold) before declining on Days 14 and 15. The activity again started increasing from Day 16 with a much smaller surge on Day 18 and was again decreasing by Day 20 of pregnancy. One-way analysis of variance indicated significant effect of gestational age on the formation of 15α-OH-E_2 (from Day 11–Day 15: $P < 0.001$, from Day 15–Day 20: $P = 0.01$).

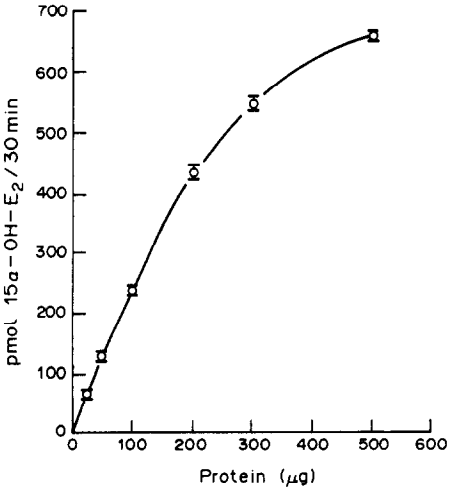

Fig. 4. Estradiol 15α-hydroxylase activity as a function of the amount of mitochondrial–microsomal protein. Blastocyst (Day 13) proteins at different concentrations were incubated as described in "Materials and Methods". Reactions were terminated after 30 min and the amounts of ^{14}C-labeled 15α-hydroxy-estradiol formed were determined as described in "Materials and Methods". Each point represents the average of three determinations (Mean ± SEM).

Table 1. Influence of SKF-525A and rabbit anti-NADPH cytochrome *P*450 reductase on E_2-15α-H activity of Day 13 pig blastocysts

Compound	% Inhibition
SKF525A:	
325 μM	44.0
650 μM	46.1
1300 μM	55.0
Anti-NADPH cytochrome *P*450 reductase:	
200 (μg/150 μl)	38.0
400 (μg/150 μl)	64.8
800 (μg/150 μl)	76.7

The values are expressed as percentage inhibition of E_2-15α-H compared to control samples. The term "inhibitor" means the ability of the test compounds to inhibit the production of radiolabelled 15α-OH-E_2. The amount of the product formed in each case was compared to control samples containing no inhibitor and run simultaneously. The control samples for antibody experiments were run with an equivalent amount of normal rabbit IgG.

Table 2. Inhibition of E_2-15α-H activity of Day 13 pig blastocysts by different steroidal and non-steroidal compounds

Compound	% Inhibition 10 μM	% Inhibition 100 μM
Progesterone	46.49	82.0
Pregnenolone	54.0	76.7
Cholesterol	4.0	4.0
Estrone	43.5	73.9
Estriol	56.5	77.9
2-Hydroxyestradiol	30.0	75.0
4-Hydroxyestradiol	11.3	46.2
2-Methoxyestradiol	59.3	87.0
Testosterone	38.5	49.2
Androstenedione	52.6	49.5
4-Hydroxyandrostenedione	16.7	16.0
2-Bromoestradiol	76.8	87.0
2-Fluoroestradiol	70.7	94.2
Diethylstilbestrol	45.0	90.0
Hexestrol	40.0	65.0

The values are expressed in the same way as in Table 1. The amounts of the products formed in two determinations were averaged and compared to control samples containing no inhibitor run simultaneously, with variability equal to or less than 6% in each case.

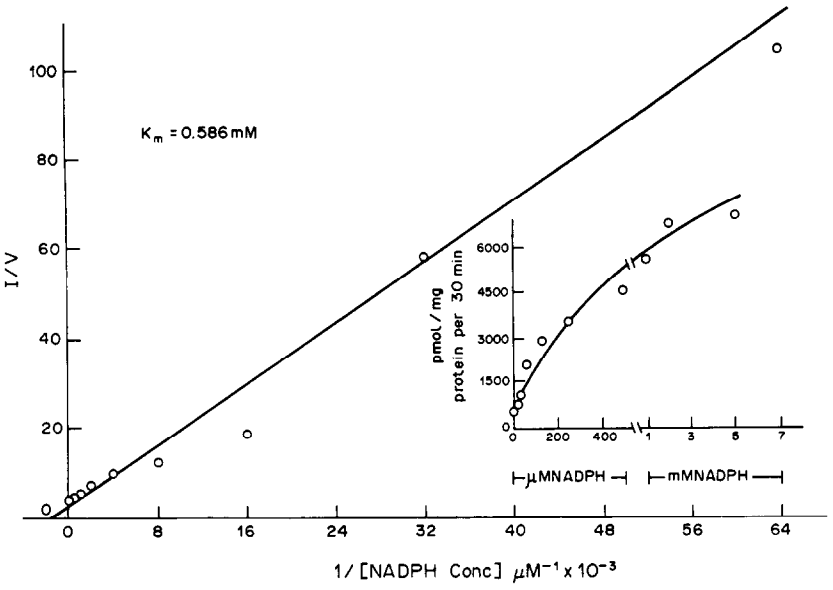

Fig. 6. Dependence of estradiol 15α-hydroxylase on the concentration of NADPH. The formation of 15α-hydroxyestradiol by the mitochondrial–microsomal fraction of pig blastocyst (Day 13) with 10 μM of [^{14}C]estradiol and various NADPH concentrations (15.625–5000 μM) is plotted on a double reciprocal plot. The apparent K_m for NADPH for the formation of 15α-hydroxyestradiol is 586 μM. Inset, formation of 15α-hydroxyestradiol, expressed as pmols per mg protein/30 min is plotted directly as a function of NADPH concentration. Each point represents the average of two determinations.

Tissue localization of E_2-15α-H

Blastocysts (Day 13) from one animal were separated into embryonic and extraembryonic tissues and 15α-OH-E_2 forming ability was measured in these two tissues. During 30 min incubations, two pools of embryonic discs produced 150 and 80 pmols 15α-OH-E_2/mg protein and extraembryonic membranes produced 9.3 and 8.06 nmols 15α-OH-E_2/mg protein. Blastocysts (Day 20) collected from several pigs were separated into embryo, allantois, yolk sac and trophoblast. E_2-15α-H activity was more concentrated in all of the extraembryonic than in the embryonic tissues ($P < 0.01$) (Table 3).

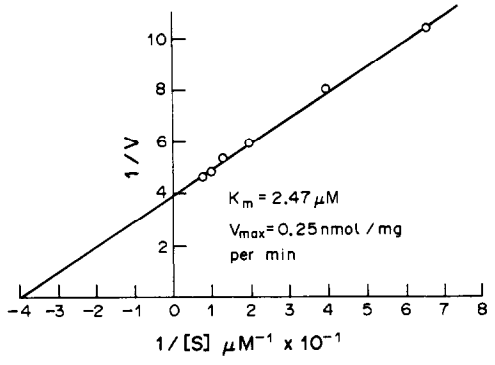

Fig. 7. Dependence of the formation of 15α-hydroxyestradiol by the mitochondrial-rich fraction on the concentration of substrate, estradiol. Various concentrations of estradiol (1.5–12.5 μM; 50,000 ^{14}C cpm/assay) were incubated with NADPH (1.5 mM) and 10,000 *g* pellet of 1000 *g* supernatent fluid of blastocyst from Day 13 of pregnancy (60 μg of protein) in a reaction volume of 300 μl for 30 min at 37°C. The [^{14}C]15α-hydroxyestradiol produced was isolated and the amount formed was quantitated as described in "Materials and Methods". The velocity of enzymatic reaction is expressed as nmol of product formed/mg of the 10,000 *g* pellet protein/min of incubation time. A double reciprocal plot of velocity versus substrate concentration yielded a linear Lineweaver–Burk plot and the data were analyzed on computerized least squares regression. The apparent K_m for estradiol of 2.47 μM and an apparent V_{max} of 0.25 nmol/mg/min were determined.

DISCUSSION

The results of the present investigation are significant for several reasons: (i) to our knowledge, this is the first report of biochemical characterization of E_2-15α-H; (ii) this enzyme is one of the native forms

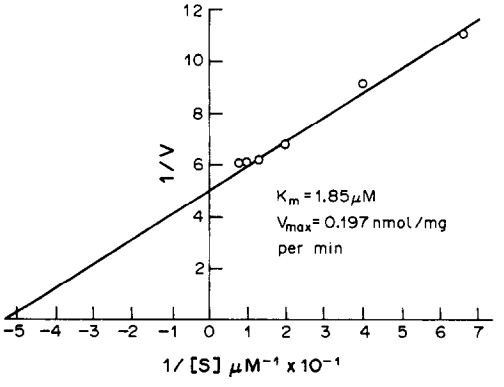

Fig. 8. Lineweaver–Burk plot for estradiol from microsomal fraction of the blastocyst. The experimental conditions were similar to those mentioned in Fig. 7. Reactions with the microsomal fractions yielded an apparent K_m for estradiol of 1.85 μM and an apparent V_{max} of 0.197 nmol/mg/min.

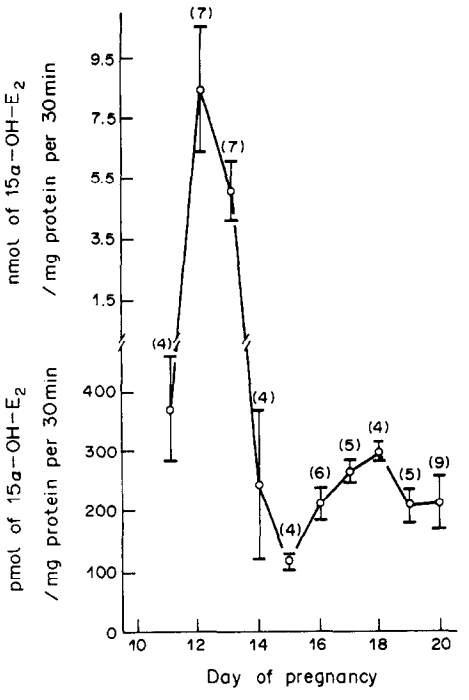

Fig. 9. Estradiol-15α-hydroxylase activity in pig blastocysts on different days of pregnancy. Aliquots of the mitochondrial–microsomal fraction (100 μg protein) were assayed for E_2-15α-hydroxylase as described in "Materials and Methods". Formation of 15α-hydroxyestradiol is expressed as nmol per mg protein per 30 min in case of Days 12 and 13 of pregnancy, and as pmols per mg per 30 min in case of the rest of the Days of pregnancy. Blastocysts from each pig were pooled and assayed separately. Each determination is again average of the results of 2–3 pools of blastocysts from each pig. The numbers in parentheses indicate the numbers of animals used for each day of pregnancy. Each point is mean ± SEM.

of cytochrome *P*450, which does not require xenobiotics for induction and is possibly being induced and regulated by endogenous steroids; (iii) 15α-OH-E_2 appears to be the one of the major products of estradiol metabolism in pig blastocysts; and (iv) stage and tissue specific distributions of the enzyme suggest a role in the establishment of pregnancy.

The kinetics of E_2-15α-H in the mitochondrial rich fraction as well as in the microsomal fraction of the pig preimplantation conceptus follow classical Michaelis–Menten kinetics. Apparent K_ms of 2.47 μM (mitochondrial-rich fraction) and of 1.85 μM (microsome) for the substrate E_2 were observed under initial velocity conditions. Under the same reaction conditions, the K_ms for the formation of 2-OH-E_2 were 3.77 μM and 1.71 μM respectively. Although K_m values are similar, the V_{max}s of E_2-15α-H are, in each case, about 8 times more than the V_{max}s of E-2-H. These observations suggest that in the pig blastocyst, 15α-hydroxylation reaction is predominant over 2-hydroxylation reaction. The existence of E-2-H and E_2-15α-H in both mitochondrial as well as microsomal fractions of the pig blastocyst is in contrast with their subcellular localization in the ovary, where both these hydroxylases are present only in the microsomal fraction (unpublished observation). Steroid hydroxylases in the liver and brain are also present only in microsomes. The process of compartmentalization of the enzymes inside the cells of the developing conceptus has not been studied. It is possible that complete compartmentilization of the enzymes occurs when the tissues are fully differentiated.

Table 3. E_2-15α-H activity in various tissues of Day 20 pig blastocysts (Mean ± SEM)

Tissue	No. of conceptuses	pmols of 15α-OH-E_2 formed per mg protein per 30 min
Total conceptus	9	215.0 ± 42.0
Trophoblast	3	410.0 ± 62.7
Yolk sac	4	90.8 ± 6.4
Allantois	8	83.7 ± 8.3
Embryo	4	31.0 ± 4.0

The findings that E_2-15α-H in the pig blastocyst requires NADPH and its activity is inhibited by CO, SKF-525A, piperonyl butoxide and antibody to NADPH cyt *P*450 reductase strongly suggest the involvement of cyt *P*450 in this hydroxylation reaction. α-Naphthoflavone specifically inhibits the cyt *P*449s, which are inducible by polycyclic hydrocarbons [17]. 15α-Hydroxylation of E_2, like 2- and 4-hydroxylations [15], was not inhibited by this agent. This suggests that cyt *P*448s cannot catalyze these hydroxylation reactions in the pig blastocyst. The degrees of inhibition of E_2-15α-H by piperonyl butoxide and anti-NADPH cyt *P*450 reductase were almost identical to those of E-2/4-H [15]. However, CO and SKF-525A inhibited E_2-15α-H with a much greater potency than they inhibited E-2-H [15]. Aminogluethimide and 4-hydroxyandrostenedione, the two inhibitors of steroid aromatase, did not inhibit E_2-15α-H activity like E 2/4 H [15]. This suggests that although there is a positive correlation between aromatase activity [17] and these hydroxylases in the pig blastocyst, identical *P*450s are not involved in catalyzing aromatization and hydroxylation reactions.

We do not know at present whether the steroidal and non-steroidal compounds which have been used to study the inhibition of E_2-15α-H are true inhibitors or alternate substrates. The results, however, provide some clue about endogenous regulations of both E-2/4-H [15] and E_2-15α-H in the pig blastocyst. As E-2/4-H, E_2-15α-H also was not inhibited by cholesterol, but was inhibited significantly by its side chain cleaved products, pregnenolone and progesterone. The inhibitory potencies of other agents were also almost similar for both E-2/4-H [15] and E_2-15α-H (Table 2). Due to the limited availability of authentic 15α-OH-E_2, we could not study the product inhibition of E_2-15α-H. Further investigation is required, however, to determine the type of inhibition by the steroids and non-steroidal estrogens, the apparent K_is of the inhibitors, and the ability of the steroids to

serve as alternate substrates of E_2-15α-H in the pig blastocyst.

The lowest activity of E_2-15α-H in pig blastocyst was noted on Day 15 of pregnancy that is similar to what was observed for E-2/4-H [18]. Stone and Seamark [19] observed increased concentrations of pregnenolone, testosterone, estrone and androstendione in plasma and uterine fluids of gilts on Day 15 of pregnancy. Because these steroids have considerable inhibitory influence on the formation of 15α-OH-E_2 in the pig blastocyst *in vitro*, the low enzyme activity on Day 15 of pregnancy may be attributed by them [19].

The two peaks of E_2-15α-H, one on Days 12–13 and the other on Days 17–18 differ both in magnitude and sharpness. The magnitude of the second peak is one-tenth that of the first peak. The first peak is formed by a sharp increase of the enzyme activity on Day 12 followed by a sharp decline on Day 14, whereas the second peak is formed by a gradual increase of the enzyme activity from Days 16 through 18 followed by its gradual decline on Day 20. These two peaks coincide with those of E-2/4-H activity in the blastocyst [7, 18], as well as several other events that occur in the blastocyst, uterus and ovary that are important for the establishment of pregnancy. The spherical blastocysts (< 1 mm in diameter) on Day 10 changes to tubular and then filamentous forms with progression of pregnancy (600 mm in length by Day 13 and up to 1 cm by Day 16). On Day 13, uniform spacing of blastocysts and their final orientations take place within the uterine horns. Attachment of blastocysts to the uterine endometrium in the pig is a gradual process and starts around Day 14 [20]. The process continues with interlocking microvilli on day 16 [20] to Day 18 [21], at the time of the second surge of E_2-15α-H. Furthermore, the abundance of this enzyme activity in the extraembryonic membranes rather than in the embryo proper both on Day 13 and Day 20 suggests its involvement in the attachment or other interactions with material tissues. In contrast, E-2/4-H activity on Day 20 of pregnancy is almost evenly divided between embryonic and extraembryonic tissues [18]. Further evidence to support involvement of the 15α-hydroxylation reaction in implantation in the pig is that E_2-15α-H is absent in the fetal liver on Days 30 and 59 of pregnancies, while it is present in the extraembryonic membranes (chorioallantois) (data not shown). Fetal livers had very high activity of E_2-15α-H (data not shown) which was absent in all of the extraembryonic tissues studied. However, both fetal livers and extraembryonic tissues had E-2-H activity.

The products of estrogens are generally thought to be less estrogenic than the parent compounds. However, several functions of these products are recently being postulated. Catecholestrogens are more effective than phenolic estrogens in stimulating prostaglandin synthesis in the rat and human uterus [22, 23] and in the rabbit blastocyst and endometrium [24]. 2-Hydroxyestrogens have also been shown to be potent biological antioxidants [25]. Recently, preferential stimulation of thymidine incorporation in progesterone-primed uterine myometrial cells of mice by E_3 has been observed [26], and this has been suggested to be associated with the necessary growth of the myometrium during pregnancy to accommodate the developing fetus. 15α-OH-E_2 could serve the same purpose in pig uterine myometrial cells. Estetrol (E_4), which has been considered as a good indicator of fetal well being in humans [27–29], is produced by the fetal liver. In this case sulfated estradiol is hydroxylated at carbon 15 before it is hydroxylated at carbon 16 [14]. This is in contrast to our present findings in the pig blastocyst in which E_2 does not require to be sulfoconjugated prior to 15α-hydroxylation. Whether pig fetal liver can hydroxylate estradiol sulfate at carbon 15 is not known.

Recently, a cyt *P*450 isozyme *P*450 C-M/F has been purified from rat liver microsomes [30], and this *P*450 has been shown to be highly active in both 2- and 16α-hydroxylations of estrogens. We do not know whether the same *P*450 is catalyzing the 2- and 15α-hydroxylations of estrogens in the pig blastocyst. The high degree of correlation between E-2-H and E_2-15α-H activities in the pig blastocyst with regard to its pattern of changes with pregnancy as well as the inhibitory pattern by the *P*450 inhibitors and the steroid and non-steroidal estrogens, is an indication that both these hydroxylation reactions could be the function of the same cyt *P*450. To resolve this issue, isolation of the haemproteins from the extraembryonic tissues of the pig blastocyst and determination of the primary structures of these proteins will be required.

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