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FORMATION AND METABOLISM OF 5(10)-ESTRENE-3β,17β-DIOL, A NOVEL 19-NORANDROGEN PRODUCED BY PORCINE GRANULOSA CELLS FROM C_{19} AROMATIZABLE ANDROGENS

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SUMMARY - The biosynthesis of non-aromatic 19-norsteroids has been studied using primary cultures of porcine granulosa cells. Formation of 5(10)-estrene-3β,17β-diol, a novel 19-norsteroid, from androstenedione and 19-hydroxyandrostenedione by porcine granulosa cells is reported for the first time. The structure was deduced from (i) comparison of its elution times on C_{18} reverse phase HPLC with authentic 5(10)-estrene-3β,17β-diol (ii) identification with 5(10)-estrene-3β,17β-diol-diacetate after acetylation (iii) oxidation/acid catalysed isomerization to 19-norandrostenedione.

Serum or serum plus FSH significantly stimulated (seven fold increase) formation of 5(10)-estrene-3β,17β-diol from androstenedione and 19-hydroxyandrostenedione. Formation of 5(10)-estrene-3β,17β-diol from both substrates was significantly ($p<0.01$) reduced by the aromatase inhibitors 4-hydroxyandrostenedione (15 μM) and aminoglutethimide phosphate (10^{-4}M). These results suggest that 5(10)-estrene-3β,17β-diol (and 19-norandrostenedione) may be formed by enzymes similar to the aromatase complex required for estradiol-17β biosynthesis.

5(10)-Estrene-3β,17β-diol is converted by granulosa cells to four metabolites. 19-Norandrostenedione was identified by crystallization to constant specific activity; 19-nortestosterone is a minor product. Production of 19-norandrostenedione and 19-nortestosterone indicates that granulosa cells possess the enzymes necessary for the transformation of 5(10)-estrene-3β,17β-diol and other 3-hydroxy-5(10)-estrenes to 19-nor-4-ene-3-ketosteroids. The formation of 5(10)-estrene-3β,17β-diol and 19-norandrostenedione as substantial metabolites of androstenedione suggest a physiological role for these 19-norsteroids in ovarian follicular development.

The high concentrations of 19-norandrostenedione (19-norA) in equine (1) and porcine (2) follicular fluid suggest that these steroids may be involved

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in the regulation of follicular function. 19-NorA, like testosterone, can act in synergism with dibutyryl cAMP to inhibit germinal vesicle breakdown in porcine oocytes (3). Porcine granulosa cells can convert androstenedione (A) and other C_{19} aromatizable androgens (4,5) to 19-norA and estradiol-17β (E_2-17β) in approximately equal quantities. Since 19-norA and E_2-17β formation is blocked by the aromatase inhibitors 4-hydroxyandrostenedione (4-OHA) and aminoglutethimide phosphate (AGP) (6), we have proposed that the biosynthesis of 19-norA from aromatizable androgens may be related to that of estrogens.

This proposal requires that 5(10)-estrene-3,17-dione is the probable intermediate formed from 19-hydroxyandrostenedione (19-OHA) or 19-oxoandrostenedione (19-oxoA) by a Retro-aldol type elimination, which is either isomerised to 19-norA or further metabolised.

We report here the identification of 5(10)-estrene-3β,17β-diol, a metabolite of 5(10)-estrene-3,17-dione which is formed when porcine granulosa cells are incubated with either A or 19-OHA.

The synthesis of 5(10)-estrene-3β,17β-diol is reduced by 4-OHA and AGP, at concentrations which inhibit formation of E_2-17β and 19-norA by porcine granulosa cells. The metabolism of 5(10)-estrene-3β,17β-diol was also investigated; this steroid is converted to 19-norA and 19-nortestosterone (19-norT). A preliminary report of some of these findings has been made (7).

MATERIALS AND METHODS

[1,2,6,7-^{3}H]-A (85 Ci/mmol), [6,7-^{3}H]-19-OHA (56 Ci/mmol) and [4-^{14}C]-A (52 mCi/mmol) were obtained from New England Nuclear, Montreal, Canada. Steroid standards were purchased from Steraloids Inc. (Wilton, NH), Research Plus Inc. (Bayonne, NJ), Sigma (St. Louis, MO) or Fluka (Ronkonkoma, NY). 5(10)-Estrene-3β,17β-diol was a gift of Dr. M. B. Groen, Organon, Holland. 4-OHA was synthesized from A (8), and AGP was a gift of Dr. Charles A. Brownley, Ciba-Geigy, Summit, NJ. Ovine FSH (NIAMDD-o-FSH-S14) was provided by the Pituitary Hormone Distribution Program, NIAMDD, while heat inactivated porcine serum was obtained from Flow Laboratories, McLean, VA.

Cell preparation and incubation

Granulosa cells (500,000 per well) from 4-6 mm follicles of prepubertal gilts were cultured in 1 ml Dulbecco's Modified Eagle's Medium in 24-well tissue culture plates (Falcon Plastics, Los Angeles, CA), at 37^{o}C in a humidified atmosphere of 5% CO_2 and 95% air for 48 h (6); 0.5 μCi [^{3}H]-A, or 0.4 μM (0.02 μCi) [4-^{14}C]-A was added to each well.

Steroid purification and HPLC analysis

Culture medium was extracted with methylene chloride (3x10 ml) and purified by Lipidex 5000 chromatography (2). Steroids were analysed by HPLC on a C_{18} μBondapak column (300 x 3.9 mm, Waters Associates, Canada) eluted isocratically with 70% methanol/H_2O (system 1) and on a C_{18} RAD PAK (10 μ, 5 mm I.D.) eluted with 40% acetonitrile/H_2O (system 2), flow 1 ml/min. HPLC equipment used has been previously documented (2). Fractions (0.25 ml) were collected and radioactivity determined in 2.5 ml liquid scintillation cocktail on an Isocap 300 counter (Nuclear Chicago). Counting efficiency for [^{3}H] and [^{14}C] were 38% and 76% respectively. 3-Oxo-4-ene steroids and

5(10)-estrenes were detected at 254 nm and 214 nm respectively by UV absorbance spectroscopy.

Isolation of [^{14}C]-5(10)-estrene

Medium from granulosa cells incubated with [^{14}C]-A (144 wells, 0.02 μCi/well) was used to isolate the [^{14}C]-5(10)-estrene for metabolism studies. The peak corresponding to authentic 5(10)-estrene-3β,17β-diol (r.t. 11.50 on HPLC system 1) was purified to homogeneity, before being used as substrate.

Metabolism of[^{14}C]-5(10)-estrene

Granulosa cells were incubated with isolated [^{14}C]-5(10)-estrene (21,500 dpm/well) for 48 h with FSH and/or serum. Culture medium was processed as described.

(a) Acetylation

Pyridine (100 μl) and acetic anhydride (500 μl) were added to [^{14}C]-5(10)estrene (15,000 dpm) and reacted overnight at room temperature. Ethanol (1 ml) was added to remove excess anhydride and the mixture dried under N_2 prior to HPLC.

(b) Jones oxidation

[^{14}C]-5(10)-Estrene (21,000 dpm) was dissolved in acetone (400 μl, 4°C) and Jones Reagent (CrO_3/H_2SO_4, 10 μl) was added (9). The reaction was stopped after 10 min with excess methanol, the mixture dried under N_2, and extracted with methylene chloride (3x5 ml). This organic extract was filtered after drying over anhydrous Na_2SO_4 and dried prior to HPLC.

(c) Jones oxidation/oxalic acid isomerization

The dried methylene chloride extract obtained following oxidation was dissolved in 4 ml 95% ethanol and anhydrous oxalic acid (5 mg) was added. The mixture was refluxed for 15 min, cooled, evaporated to dryness, extracted with methylene chloride (3x1.5 ml), and the organic extract dried prior to HPLC.

Statistical Analysis

Experiments were replicated four times. Data from each experiment were combined and recorded as mean $\pm$ SEM for replicate analyses. Data were compared using one-way analysis of variance (ANOVA) and Duncan's multiple range test was used to ascertain significant differences among individual means.

RESULTS

Metabolism of [^{3}H]-androstenedione by granulosa cells

Serum or serum plus FSH significantly ($p<0.01$) stimulated formation (7-fold increase) of 5(10)-estrene-3β,17β-diol from A and 19-OHA over controls and treatments with FSH (Figure 1). 19-NorA and E_2-17β formation was also significantly ($p<0.05$) stimulated by serum, and by serum plus FSH, from both substrates.

5(10)-Estrene-3β,17β-diol synthesis from A and 19OHA was significantly ($p<0.01$) inhibited by 4-OHA (15 μM) and AGP (10^{-3}M, 10^{-4}M) (Figure 1) when

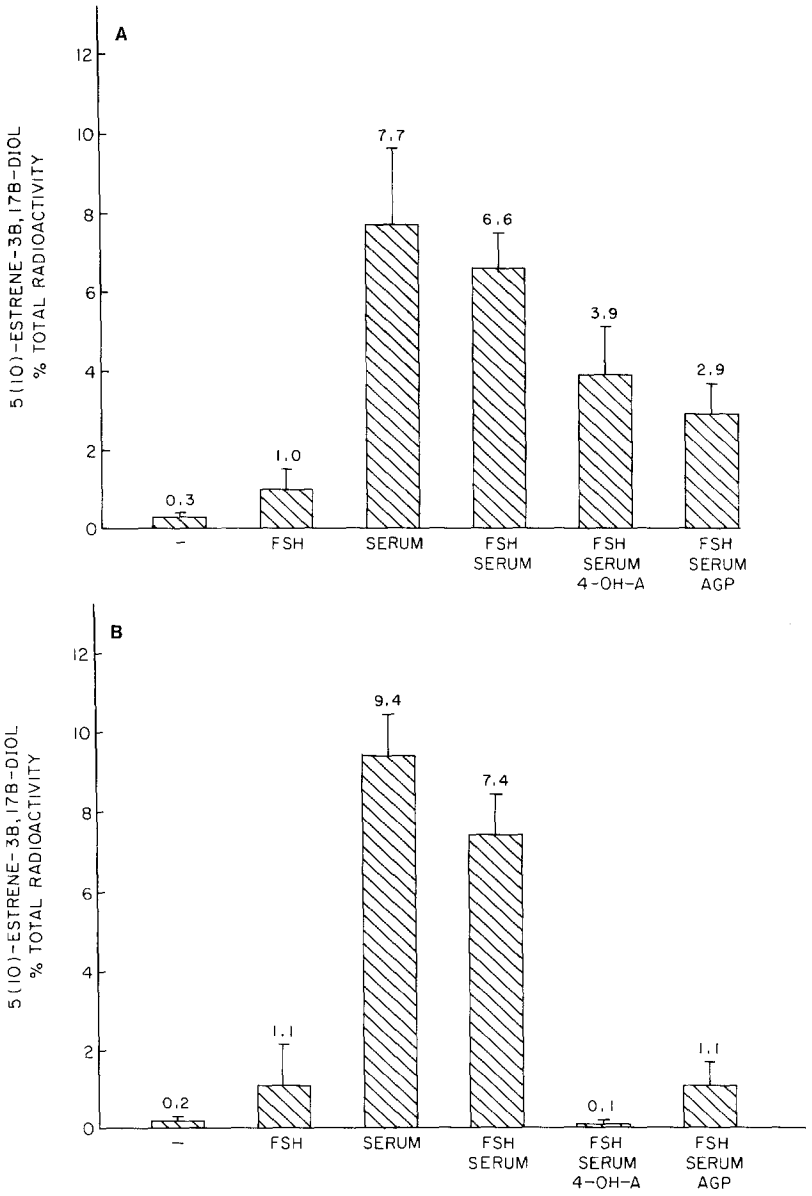

Figure 1. Formation of 5(10)-estrene-3β,17β-diol from androstenedione (Figure 1A) and 19-hydroxyandrostenedione (Figure 1B) by porcine granulosa cells with and without FSH and serum. Concentrations of each steroid are expressed as the percentage of total radioactivity, and represent the mean $\pm$ SEM of four wells for each treatment from four independent experiments. 5(10)-Estrene-3β,17β-diol formation from A was significantly inhibited ($p<0.01$) by 4-OHA (15 μM) and AGP (10^{-4}M). Formation from 19-OHA was significantly inhibited ($p<0.01$) by 4-OHA (1 μM) and AGP (10^{-5}M).

steroid formation was maximally stimulated by serum and FSH. 5(10)-Estrene-3β,17β-diol formation from 19-OHA was also significantly inhibited ($p<0.01$) at concentrations as low as 10^{-6}M 4OHA and 10^{-5}M AGP.

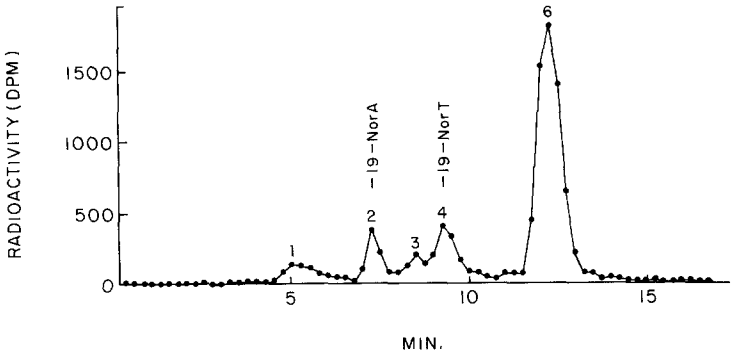

Figure 2. HPLC chromatogram (system 1) of steroid metabolites produced by porcine granulosa cells incubated with [4-^{14}C]-5(10)-estrene-3β,17β-diol. Peak 2 is 19-norA; 3 is 19-norT; 5 is unmetabolized substrate; 1 and 4 are unidentified metabolites.

(a) Acetylation

HPLC analysis of the 5(10)-estrene gave a single product whose retention time (r.t.) (7.0 min) on C_{18} μBondapak (95% methanol/H_2O) was similar to that of authentic 5(10)-estrene-3β,17β-diol diacetate.

(b) Oxidation

Jones oxidation of the isolated 5(10)-estrene gave four products; 19-norA (27%) was the major product obtained from HPLC (systems 1 and 2).

(c) Oxalic acid isomerization/Jones oxidation

In two experiments 19-norA (27%) and 19-norT (8%) were obtained in addition to other uncharacterized metabolites with r.t.'s (HPLC system 1) of 4.5, 5.25 and 28.5 min.

Metabolism of [^{14}C]-5(10)-estrene-3β-17β-diol by granulosa cells

Granulosa cells with serum and FSH converted [^{14}C]-5(10)-estrene-3β,17β-diol to four metabolites, two of which corresponded to 19-norA (4.8%) and 19-norT (10.7%) (Figure 2). The 19-norA peak (23,000 dpm) was isolated by HPLC and recrystallized to constant specific activity with 36 mg authentic unlabelled 19-norA (Table 1).

DISCUSSION

The mammalian ovarian follicle consists of two cell types, the theca and granulosa. Granulosa cells stimulated by FSH, can form estrogens from C_{19} aromatizable androgens supplied by the theca (10). In addition to estrogens, porcine granulosa cells make 19-norA (6) and 5(10)-estrene-3β,17β-diol. Formation of these steroids is inhibited by 4-OHA and AGP at concentrations which inhibit estrogen synthesis in other systems (11).

Formation of 5(10)-estrene-3β,17β-diol by granulosa cells provides evidence for the involvement of 5(10)-estrene-3,17-dione as a potential

Table 1

Crystalization of the radioactive peak corresponding to 19-norA obtained from granulosa cell metabolism of [4-^{14}C]-5(10)-estrene-3β-17β-diol with authentic unlabelled 19-norA

Number of Crystalizations	Specific Activity (dpm/mg) Crystal	Supernatant
1	318	1033
2	301	905
3	298	982
4	294	459
5	285	318

Authentic unlabelled 19-norA used = 0.036 g

The specific activity (crystal) of 4th and 5th crystallizations is constant (difference ~3%)

precursor in 19-norA biosynthesis. These results indicate that in the porcine follicle, C_{19} aromatizable androgens produced by the theca, are converted to 19-norsteroids by granulosa via 5(10)-estrene intermediates. A similar pathway may exist in the horse since equine testis homogenates produce 5(10)-estrene-3,17-diols of unspecified stereochemistry (at C3 and C-17) from dehydroepiandrosterone (12). Sulfo-conjugates of 5(10)-estrenes have also been reported in stallion urine (13).

The formation of 19-norA and 19-norT is consistent with the assumption that porcine granulosa cells possess the enzymes necessary for the transformation of 5(10)-estrene-3β,17β-diol and other 3-hydroxy-5(10)-estrenes to 19-nor-4-ene-3-ketosteroids. A likely enzyme system, active in porcine granulosa cells in converting pregnenolone to progesterone, is the 3β-hydroxysteroid dehydrogenase/isomerase. This enzyme complex in the bacterium, Pseudomonas testosteroni, converts 5(10)-estrene-17β-ol-3-one to 4-estrene-17β-ol-3-one (19-norT), albeit at slower rates compared to conversion of 5-androstene-3,17-dione to 4-androstene-3,17-dione (14).

The precise reproductive function of endogenous 19-norsteroids in the pig (2) and the horse (1,12,13) is unknown. As androgens, they can regulate the activity of the 3β-HSD/isomerase and affect progesterone synthesis by granulosa cells (15). As competitive inhibitors of the placental aromatase complex, 19-norsteroids can also affect estrogen biosynthesis (16).

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REFERENCES

1. Short, R. (1960) Nature 188: 232.
2. Khalil, M.W., and Walton, J.S. (1985) J. Endocrinol. 107: 375-381.
3. Daniel, S.A.J., Khalil, M.W., and Armstrong, D.T. (1986) Gamete Research 13: 173-184.
4. Garrett, W.M., Kettelberger, D., Hoover, D., Shackleton, C.H., and Anderson, L.D. (1986) Society for the Study of Reproduction, 19th Annual Meeting, abstract 44.
5. Khalil, M.W., Morley, P., Glasier, M.A., and Armstrong, D.T. (1987) Steroids 50: (In press).
6. Khalil, M.W., Morley, P., Glasier, M.A., Armstrong, D.T., and Lang, T. (1988) J. Endocrinol. (Submitted).
7. Khalil, M.W., Morley, P., Glasier, M.A., and Chung, N. (1988) Endocrine Society, 70th Annual Meeting, abstract 456.
8. Brodie, A.M.H., Schwarzel, W.C., Shaikh, A.A., and Brodie, H.J. (1977) Endocrinology 100: 1684-1695.
9. Djerassi, C., Engle, R.R., and Bowers, A. (1956) J. Org. Chem. 21: 1547-1549.
10. Leung, P.C.K. and Armstrong, D.T. (1980) Ann. Rev. Physiol. 42: 71-82.
11. Brodie, A.M.H. (1985) Biochem. Pharmacol. 34: 3213-3219.
12. Smith, S.J., Cox, J.E., Houghton, E., Dumasia, M.C. and Moss, M.S. (1987) J. Reprod. Fert., Suppl. 35: 71-78.
13. Houghton, E., Teale, P. and Dumasia, P. (1984) Biomed. Mass Spectrom. 11: 96-99.
14. Talalay, P. and Wang, V.S. (1955) Biochim. Biophys. Acta 18: 300-301.
15. Lischinsky, A., Evans, G. and Armstrong, D.T. (1983) Endocrinology 113: 1999-2003.
16. Schwarzel, W.C., Kruggel, W.G. and Brodie, H.J. (1973) Endocrinology 92: 866-879.