Revista Científica, FCV-LUZ / Vol. XXII, Nº 2, 103 - 111, 2012

ROLE OF ENDOTHELIN 1 IN STEROID PRODUCTION BY OVINE GRANULOSAL CELLS COLLECTED FROM LARGE PREOVULATORY FOLLICLES

Rol de la Endotelina 1 en la Producción de Esteroides por Células de la Granulosas Colectadas de Folículos Preovulatorios Ovinos

Fernando Perea Ganchou[1*], Emmett Keith Inskeep[2] y Jorge Antonio Flores[3]

*[1]Departamento de Ciencias Agrarias, Universidad de los Andes, Trujillo, Venezuela. [2]Division of Animal and Nutritional Sciences, Davis College of Agriculture, Forestry and Consumer and [3]Department of Biology, Eberly College of Arts and Sciences. West Virginia University, Morgantown 26506. *E-mail: ferromi@ula.ve*

ABSTRACT

For this experiment it was hypothesized that endothelin 1 (END1) acting through its receptors, endothelin receptor type A (ENDRA) and/or endothelin receptor type B (ENDRB), on granulosal cells (GC) of ovine preovulatory follicles would inhibit steroid production, and therefore, prevent the premature luteinization of granulosal cells of those follicles. The ovaries of 17 Suffolk ewes were removed by mid-ventral laparotomy after two doses of $PGF_{2\alpha}$ (5 mg; three h apart). Granulosal cell were harvested under sterile conditions from the two or three largest follicles. Aliquots of 15 µL of GC suspension from each presumed preovulatory follicle were cultured in 96-well plates containing a solution of 185 µL of Ham's F12 supplemented medium and treatments (single and combined effects of LH, END1, ENDRA and ENDRB). According to estradiol (E_2) concentration in follicular fluid (FF), presumed preovulatory follicles were categorized in estrogen-active and estrogen-inactive follicles. Data from 20 estrogen-active follicles (collected from 13 ewes) challenged to 10 different treatments were examined by analysis of variance. Accumulation of E_2 and progesterone (P_4) by cultured GC was not affected by either LH or END1 or the END receptor blockers. Granulosal cells from follicles with the greatest concentration of E_2 in FF produced on the average significantly more E_2 and P_4 than follicles with lower concentrations of E_2 in FF (P<0,0001). In conclusion, under the experimental conditions of this study, END1 did not affect steroid production by GC from preovulatory follicles in sheep, and therefore the working hypothesis was not supported.

Key words: Preovulatory follicle, endothelin 1, granulosal cells, progesterone, estradiol, sheep.

RESUMEN

En este experimento fue hipotetizado que la endotelina 1 (END1) actuando a través de sus receptores tipo A (ENDRA) y/o tipo B (ENDRB) en células de la granulosa obtenidas de folículos ovulatorios, inhibiría la producción de esteroides previniendo la prematura luteinización de las células de la granulosa (CG). Los ovarios de 17 ovejas Suffolk fueron removidos mediante laparotomía ventral, luego de dos dosis de $PGF_{2\alpha}$ (5 mg; tres horas de intervalo entre dosis). Las CG fueron colectadas bajo condiciones de asepsia de los dos o tres folículos más grandes. Alícuotas de 15 µL de CG en suspensión, de cada presunto folículo preovulatorio, fueron cultivadas en placas de 96 hoyos que contenían una solución de 185 µL de medio suplementado Ham's F12 más los tratamientos (efectos individuales o combinados de LH, END1, ENDRA y ENDRB). De acuerdo a la concentración de estradiol (E_2) en el fluido folicular (FF), los presuntos folículos preovulatorios fueron clasificados en estrogénicamente activos o inactivos. Los datos de la producción de E_2 y de progesterona (P_4) en el medio de cultivo de 20 folículos estrogénicamente activos sometidos a 10 tratamientos diferentes se analizaron mediante análisis de varianza. La concentración acumulada de E_2 y P_4 en el medio luego de 24 h de cultivo no fue afectada por la LH, ni END1, ni por los antagonistas de los receptores de END1 y sus combinaciones. Las CG colectadas de folículos con mayor concentración de E_2 en el FF produjeron en promedio mayor cantidad de E_2 y P_4 que los obtenidos de folículos con menor concentración de estradiol (P<0,0001). En conclusión, bajo las condiciones experimentales aplicadas en este estudio, END1 no afectó la producción de esteroides por las células de la granulosa obtenidas de folículos preovulatorios ovinos, y por lo tanto la hipótesis no fue cofirmada.

Palabras clave: Folículo preovulatorio, endotelina 1, células de la granulosa, progesterona, estradiol, ovejas.

Recibido: 07 / 04 / 2011. Aceptado: 10 / 12 / 2011.

INTRODUCTION

In sheep (*Ovis aries*), follicular growth occurs in a wave-like pattern with typically three to four follicular waves during every estrous cycle [22]. Follicular dominance is not as marked in sheep as it is in the cow (*Bos taurus-Bos indicus*) [24, 39]. Neither the presence of a large follicle induced by exogenous follicular stimulating hormone (FSH) nor supraphysiological concentrations of estradiol released from implants prevented emergence of a new wave of follicles [6, 14]. Likewise, more than two antral follicles from ultimate and penultimate waves of the cycle may reach ovulatory status and eventually ovulate [7, 44].

Preovulatory follicles produce greater amounts of estradiol [7], and elevated concentrations of circulating estradiol trigger the gonadotropin surge and the consequent ovulation process [38]. After the luteinizing hormone (LH) surge, the predominant pattern of steroid production by the preovulatory follicles changes rapidly from estrogen (E_2) to progesterone (P_4) as the main steroid secreted by granulosal and thecal cells [20, 40]. The downregulation of P450-17α-hydroxylase and P450 aromatase in thecal and granulosal cells, respectively, and upregulation of P_4 receptor in the two types of steroidogenic cells appear to support this change [34, 54]. Likewise, steroid production by granulosal and thecal cells of large follicles is regulated by numerous factors through autocrine and paracrine actions: insulin [45], bond morphogenesis proteins (BMPs) [10], BMP15 [35], growth differentiation factor 9 (GDF9) [47], inhibin [33], estradiol [46], insulin growth factors (IGFs) [45], and endothelin 1 (END1) [1, 16]. These factors modulate steroid production through autocrine and paracrine mechanisms, and might prevent premature differentiation of granulosal and thecal cells of preovulatory follicles prior to LH/FSH surge.

Endothelin 1, a 21-amino acid peptide was described originally as a potent vasoconstrictor [56] and later associated with modulation of steroidogenesis in follicular and luteal tissue from rats [49, 50], pigs (*Sus scrofa domestica*) [16, 30, 36, 37], cows [1, 23], ewes [13] and human beings (*Homo sapiens*) [11,12]. Endothelin 1 is synthesized and secreted from ovarian granulosal cells, and greater amounts of END1 were detected in larger than in smaller antral follicles [19, 30]. The antisteroidogenic effect on granulosal cells appeared to be mediated via endothelin receptor type A (ENDRA) in pigs [17] and endothelin receptor type B (ENDRB) in rats [31]. The END1 mRNA expression in pigs was detected first around the antral stage, increased as the follicles grew, and decreased rapidly after ovulation [19]. In the rat (*Rattus rattus*) ovary, inhibition of P_4 production in granulosal cells by END1 was associated with downregulation of cholesterol side-chain cleavage enzyme (P450scc) and 3-beta-hydroxysteroid dehydrogenase (3β-HSD) and upregulation of 20-alpha-hydroxysteroid dehydrogenase (20α-HSD), 5α-reductase and 3-alpha-hydroxysteroid dehydrogenase 3α-HSD [49], enzymes implicated in synthesis and metabolism of P_4, respectively. Moreover, in bovine microdialyzed mature follicles, END1 inhibited P_4 secretion and stimulated E_2 secretion [1, 2]. Although there is no evidence of a role of END1 in regulating steroidogenesis in sheep follicles, on the basis of previous experimental evidence, it is possible to speculate that END1 acting through autocrine and paracrine mechanisms, might prevent premature luteinization of granulosal cells prior to the LH surge in sheep as suggested in other species [19, 49].

The objective of this study was to examine the effect of blocking ENDRA and ENDRB on follicular steroid production by granulosal cells from preovulatory follicles in sheep. The hypothesis was that END1 acting through ENDRA and/or ENDRB would inhibit steroid production, and therefore, prevent the premature luteinization of granulosal cells of ovine preovulatory follicles.

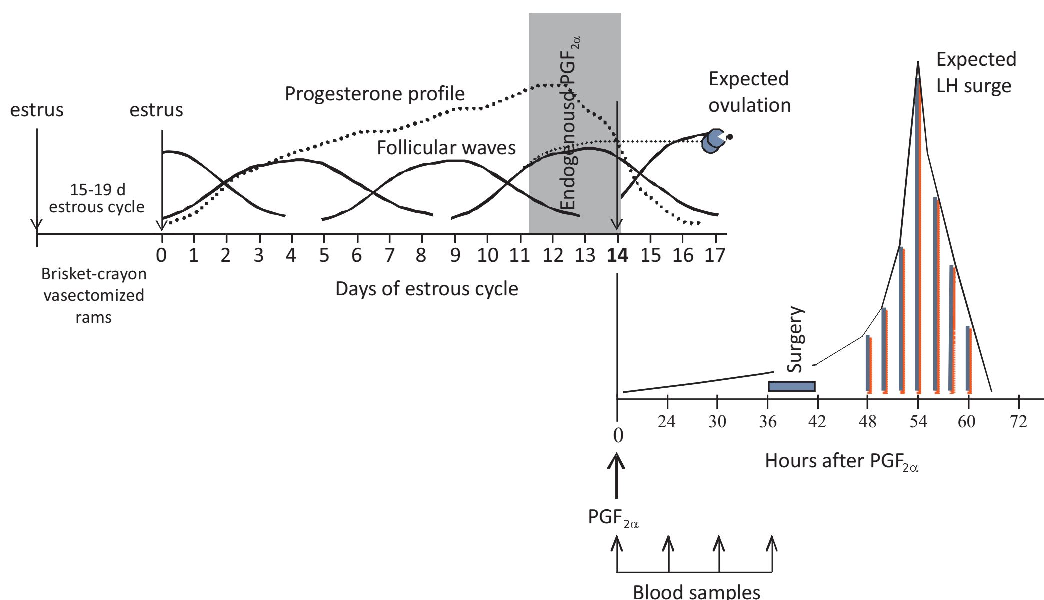

FIGURE 1. PROTOCOL FOR COLLECTION OF PREOVULATORY FOLLICLES IN SHEEP.

MATERIALS AND METHODS

General experimental procedure

Seventeen nonpregnant cycling Suffolk ewes (range 60-100 kg) with at least one previous estrous cycle of normal length (15 to 19 days) were used in the experiment, conducted in fall of 2006 (September-December) with ewes from the West Virginia University research flock. For observation of estrus (12 h intervals), ewes were penned with a vasectomized ram bearing a harness with a crayon in the area of the brisket; standing estrus was confirmed by teasing with another vasectomized ram. Fourteen days after estrus (estrus = day 0) ewes were injected (i.m.) with two doses (5 mg each) of $PGF_{2\alpha}$ (Lutalyse®, Pfizer Animal Health, New York, NY-USA) three h apart [27] to induce luteolysis. To verify that a surge of LH had not occurred before collection of follicles, jugular blood samples (8 mL) were collected at h -3, 0, 6, 12, 18, 24, 30 and 36 in relation to the second dose of $PGF_{2\alpha}$ (FIG. 1). Blood samples were refrigerated (Whirlpool, OZR134A, Canada) for 24 h to allow them to clot, and then centrifuged (International Equipment Company, IEC, Centra-7R, USA) for 20 minutes at 1400 χg. Serum was collected and kept at -20°C until radioimmunoassay (Multipurpose Scintillation Counter, USA) for LH.

Thirty six to 40 hours after the second dose of $PGF_{2\alpha}$, ewes were anesthetized with 0.3 mg/kg diazepam (Valium, 5 mg/mL; Roche Pharmaceutivals, Nutley, NJ-USA) and 7 mg/kg ketamine (Vetamine, 100 mg/mL; Mallinckrodt Veterinary, Mundelein, IL-USA), and then placed on a mixture of halothane (Halocarbon Laboratries, Riveredge, NJ-USA), oxygen (2.0 L/min) and nitrous oxide gas (1.0 L/min) until bilateral ovariectomy was carried out through a mid-ventral incision. Immediately after ovariectomy, ovaries of each ewe were placed in cold phosphate buffered saline (PBS) and stored on ice until completion of the surgery. Follicles were dissected and granulosal cells were harvested. All animal procedures were approved by West Virginia University Institutional Animal Care and Use Committee (ACUC # 05-1205).

Culture of granulosal cells and treatments

Ovarian stroma from 28 presumed preovulatory follicles (1.7 ± 0.8 follicles/ewe) was removed and the diameter of each dissected follicle was recorded (9.2 ± 1.5 mm). Using a 22-gauge needle attached to a 3 mL syringe, follicular fluid (FF) of each follicle was aspirated and centrifuged (Centra, International Equipment Company, Nashville, TN-USA) at 400 × g at 4°C for 5 min and the supernatant stored at -20°C (Isotemp Freezer, Fisher Scientific, Suwanee, GA-USA) for subsequent steroid determination by RIA; the remaining granulosal cells were added to those granulosal cells obtained directly from the follicle (see below).

Each follicle was bisected under sterile conditions and granulosal cells were scraped gently from the basement membrane with a spatula into F12 Nutrient Mixture (Ham's F12) media supplemented with sodium bicarbonate (0.01 M), antibiotics (1.5 mg of penicillin/mL and 2.5 mg of streptomycin/mL) and 19-OH androstenedione (1 µM) [32]. Granulosal cells were rinsed in cold Ham's F12 supplemented medium, centrifuged at 400 × g for 5 min at 4°C and resuspended in 200 µL of cold Ham's F12 supplemented medium. This last procedure was repeated twice. The number of cells and cell viability were estimated using a hemocy-

TABLE I

TREATMENTS USED TO EVALUATE THE ROLE OF END1 IN STEROID PRODUCTION BY GRANULOSAL CELLS FROM PREOVULATORY FOLLICLES IN SHEEP.

	Treatments	Description	Dosages
1	Control	Ham's F12 (Sigma-Aldrich, Milwaukee, WI) supplemented media	–
2	LH	Luteinizing hormone (LH, NIADDK-oLH-25 AFP 5551B;)	150 ng/mL
3	END1	Endothelin-1 peptide (END1; Bachem Bioscience Inc, King of Prussia, PA)	100 nM
4	LH+ END1		as above
5	ENDRAa	selective END receptor type A antagonist (ENDRAa): BQ-610 (Azepane-1-carbonyl-leu-D-Trp(For)-D-Trp-OH; Bachem Bioscience Inc, King of Prussia, PA)	1500 nM
6	ENDRAa+LH+ END1		as above
7	ENDRBa	selective END receptor type B antagonist (ENDRBa): BQ-788 (N-cis-2,6-Dimethylpiperidinocarbonyl)-D-Nle-OH; Bachem Bioscience Inc, King of Prussia, PA)	1000 nM
8	ENDRBa +LH+END1		as above
9	ENDRAa+ ENDRBa	END receptor types A and B antagonist (ENDRA+B)	as above
10	ENDRAa+ENDRBa+ LH+END1		as above

tometer and trypan blue exclusion dye, respectively. According to the number of granulosal cells harvested from each follicle, an additional volume of Ham's F12 supplemented medium was added to the cell suspension. Approximately 5.9 ± 2.6 × 10^5 cells were cultured in each well. Nevertheless, because differentiating viable and non-viable cells by the trypan blue method was sometime difficult, steroid concentrations per well were normalized against total protein content per well and expressed as pg of steroid per µg of protein in 24 h. Total protein at end point of the incubation period was quantified by colorimetric method using the Coomassie Protein Assay (Pierce Biotechnology, Inc., Rockford, IL-USA) [9].

Aliquots of 15 µL of granulosal cell suspension obtained from each presumed preovulatory follicle were cultured in 96-well plates containing a solution, previously equilibrated at 37°C, of 185 µL of Ham's F12 supplemented medium and treatments (TABLE I). Cultures were incubated at 37°C in a humidified atmosphere (5% CO_2 and 95% air) for 24 h. Once finished the incubation period, the medium was removed carefully from each well, centrifuged (3000 × g for 1 min at room temperature) and stored at -20°C for subsequent steroid assays by RIA. Cultured granulosal cells and the remaining volume of granulosal cells suspension were stored at -20°C.

Radioimmunoassays for LH, progesterone, and estradiol

Concentrations of P_4 and E_2 in FF and culture media, and LH concentrations in serum were measured in duplicate by RIA as previously described [25, 42, 53]. LH assay sensitivity averaged 0.54 ng/mL, and the intraassay coefficient of variation was 15%. Intraassay coefficients of variation and sensitivities for steroids in unextracted FF were 8.6%, and 34 pg/tube for P_4 and 10.6%, and 32 pg/tube for E_2, respectively. For steroids in culture media, the inter- and intra-assay coefficients of variation and sensitivities were P_4, 12.3%, 14.4%, and 0.20 ng/mL; and E_2, 13.0%, 14.9%, and 0.25 pg/mL.

Statistical analysis

Concentrations of P_4 and E_2 in culture media were evaluated by one-way analysis of variance using the General Linear Model (GLM) procedure of SAS [48]. Differences among means were compared by the LSM procedure of SAS. Data were expressed as mean ± SEM. Antral follicles with greater concentrations of P_4 than E_2 were considered atretic [28] and estrogen-inactive (P_4>E_2) [29] and these follicles were removed from the analysis. Estrogen-active follicles (E_2>P_4) were categorized according to E_2 concentration in FF into three groups (1: ≤ 250 ng/mL; 2: > 250 ≤ 350 ng/mL, and 3: > 350 ng/mL) and included as a discrete variable in the statistical model. Two ewes had concentrations of LH greater than 180 ng/mL in samples collected at 24 or 30 h after the second dose of $PGF_{2\alpha}$ (indicative of LH surge), and their follicles were excluded from the analysis. The final number of follicles examined was 20, which were collected from 13 ewes. For nine of these follicles, cells were cultured in duplicate and the average value was utilized.

RESULTS AND DISCUSSION

Accumulation of E_2 and P_4 was not affected by either LH or END1 or the END receptor blockers (FIG. 2). Correlations between concentration of E_2 in FF and E_2 and P_4 accumulation

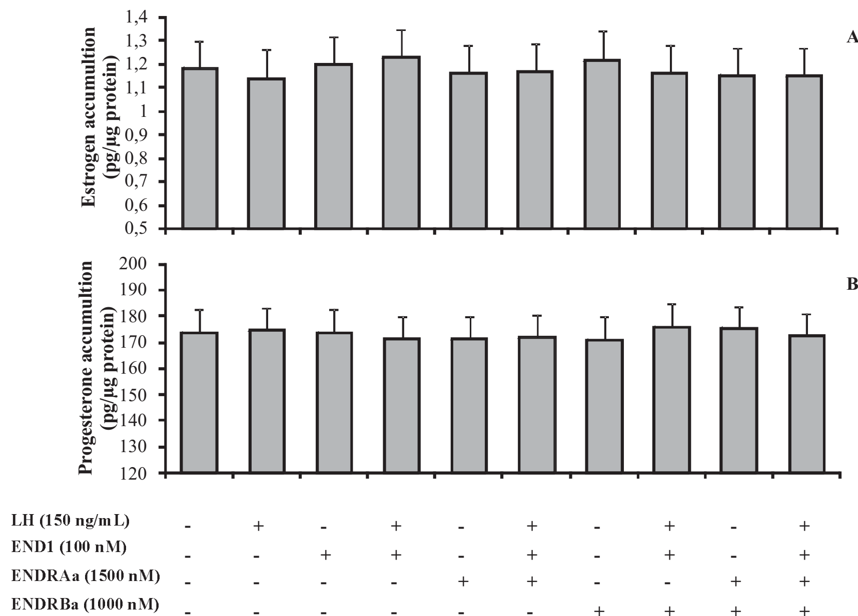

FIGURE 2. EFFECTS OF LH, END1, ENDRAa (BQ-610) AND ENDRBa (BQ-788) ON PRODUCTION OF ESTRADIOL AND PROGESTERONE BY OVINE GRANULOSAL CELLS ISOLATED FROM PREOVULATORY FOLLICLES. BARS REPRESENT MEAN ± SEM OF ESTRADIOL (PANEL A) OR PROGESTERONE (PANEL B) ACCUMULATION EXPRESSED IN pg/µg OF PROTEIN IN 24 HOURS.

TABLE II

STEROID ACCUMULATION IN MEDIA FROM CULTURED GRANULOSAL CELLS ACCORDING TO ESTRADIOL CONCENTRATION IN FOLLICULAR FLUID FROM PREOVULATORY FOLLICLES.

Steroid accumulation by granulosal cells	Concentration of E_2 in follicular fluid (ng/mL)			Significance level
	≤ 250	> 250 ≤ 350	> 350	
Estradiol	0.75 ± 0.06[a]	0.80 ± 0.08[a]	1.97 ± 0.07[b]	[a,b] P<0.0001
Progesterone	145.0 ± 3.9[a]	170.8 ± 5.9[b]	203.4 ± 7.6[c]	[a,b,c] P<0.0001

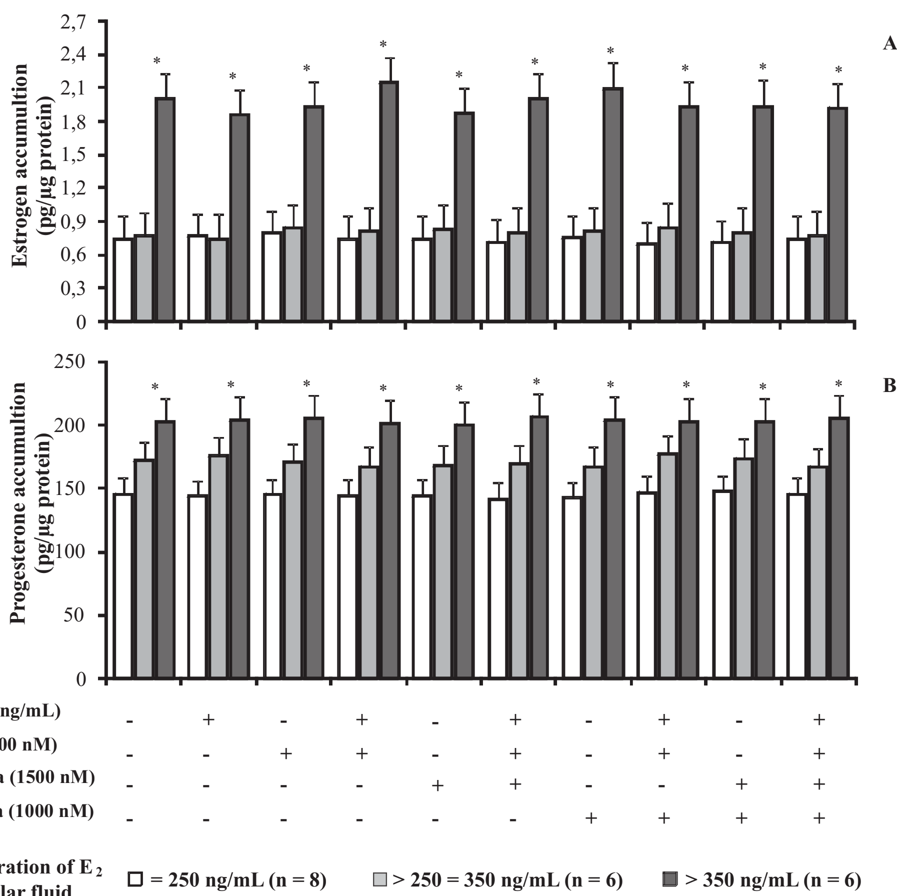

FIGURE 3. EFFECTS OF LH, END1, ENDRAA (BQ-610) AND ENDRBA (BQ-788) ON PRODUCTION OF ESTRADIOL AND PROGESTERONE BY OVINE GRANULOSAL CELLS ISOLATED FROM PREOVULATORY FOLLICLES. ACCORDING TO CONCENTRATION OF ESTRADIOL IN FOLLICULAR FLUID, PREOVULATORY FOLLICLES WERE CATEGORIZED IN THREE GROUPS (1: ≤ 250 ng/mL, 2: > 250 ≤ 350 ng/mL, AND ≥ 350 ng/ mL). BARS REPRESENT MEAN ± SEM OF ESTRADIOL (PANEL A) AND PROGESTERONE (PANEL B) ACCUMULATION EXPRESSED IN pg/g OF PROTEIN IN 24 H. VALUES WERE STATISTICALLY DIFFERENT BETWEEN GROUPS 1 AND 2 VERSUS GROUPS 3 (*P < 0.001) FOR ESTRADIOL AND BETWEEN GROUPS 1 AND 3 (*P < 0.02) FOR PROGESTERONE.

in the culture media were 0,30 (P<0,0001) and 0,26 (P<0,0004), respectively. Granulosal cells from follicles with the greatest concentration of E_2 in FF produced on the average significantly more E_2 and P_4 than follicles with lower concentrations of E_2 in FF (TABLE II). Treatments did not affect accumulation of E_2 and P_4 in any of the categories of follicles considered (FIG. 3). However, for all treatments, granulosal cells from preovulatory follicles with the greatest concentration of E_2 in FF produced greater amounts of E_2 and P_4 than preovulatory follicles with lower concentration of E_2 in FF (FIG. 3).

Granulosal cells from preovulatory follicles with greater concentrations of estradiol produced more E_2 and P_4, most likely due to the greater steroidogenic capacity of these follicles [51, 52] compared to follicles with lower estrogenic production. Dominant follicles expressed greater amounts of mRNA for LH receptor, P450scc, P450c17 and 3HSD in thecal cells and P450arom in granulosal cells than subordinate follicles [4, 5, 55]. Moreover, follicular dominance was associated with onset of expression of LH receptor and 3HSD in granulosal cells [4, 5, 8]. Hence, in the preovulatory follicles collected in this study,

greater steroid production may be supported by a greater expression of LH receptor and steroidogenic enzymes in two endocrine populations of cells. It is clear that some preovulatory follicles collected from either the same or different animals may have had functional advantages over others with lower rank. Thus, granulosal cells from those follicles reflected more steroidogenic capacity with greater accumulation of E_2 and P_4 after 24 h of culture. In addition, given that the substrate for synthesis of estradiol was provided in culture media, aromatization of androstenedione to estradiol by granulosal cells was carried out rapidly.

Unexpectedly, LH did not induce an increase of either E_2 or P_4. Evidence indicated that LH and FSH stimulated steroid production by granulosal cells in vitro and END1 prevented that increment in other species [18, 30, 37, 49]. However, responsiveness of LH receptors in follicles exposed to sustained activation by LH decreased progressively [41, 57], most likely because components of the downstream signaling pathway were disrupted [15, 26]. Aggregation of receptors and receptor internalization is another mechanism of agonist-induced desensitization of LH receptors [3, 21]. In this study, preovulatory follicles were collected at a specific follicular stage prior to LH surge, and probably were exposed to increasing concentrations of LH over time. In that circumstance, presence of elevated and sustained concentrations of LH in culture media may have induced deactivation of LH receptors in granulosal cells, and prevented stimulation of E_2 and P_4 production.

Endothelin 1, via either ENDRA or ENDRB was hypothesized to regulate follicular steroid production and prevent premature luteinization of granulosal cells before the LH surge in sheep, as suggested for other species [19, 49]. In this experiment, neither individual nor combined presence of LH and END1 in the culture medium affected the patterns of E_2 and P_4 secretion by granulosal cells isolated from preovulatory follicles. Hence, the hypothesis that END1 may modulate steroid production by granulosal cells from preovulatory follicles was not supported. Alternatively, the added androstenedione could have overshadowed detection of an effect when granulosal cells were incubated alone without thecal cells. Although anti-steroidogenic actions of END1 were demonstrated in follicles of rats [49, 50], pigs [16, 30, 37] and human beings [11, 12], such a role of END1 has not been reported in sheep follicles. In microdialyzed large bovine follicles, END1 stimulated E_2 secretion and inhibited P_4 and androgen production [1, 2]. Moreover, LH increased END1 release from mature follicles [1] and ENDRA and ENDRB were detected in the theca of bovine follicles [2]. Attempts to identify END1 by immunohistochemistry in follicles from days two to six of the ovine estrous cycle were not successful (data not shown). Because the presence of END1 in bovine preovulatory follicles was associated with other vasoactive peptides such as angiotensin II and atrial natriuretic peptide in microdialyzed mature follicles [2], a role of END1 in modulating blood flow into the follicle may be one of its functions.

Immediately after the LH surge, the pattern of steroid secretion from the preovulatory follicle is known to change abruptly [20, 40] from estrogen to P_4 dominance. It was postulated that END1, a 21-amino acid peptide with recognized anti-steroidogenic properties, might modulate steroid production preventing premature luteinization of granulosal and thecal cells [19, 49]. In this experiment the protocol designed for collection of large preovulatory follicles was successful and granulosal cells from 20 of 28 follicles were cultured and challenged to 10 different treatments, resultant from combinations of LH, END1, ENDRA and ENDRB. However, neither E_2 nor P_4 accumulation in the culture media was affected by treatments after 24 h of incubation. To interpret these findings, it is important to consider three aspects. First, granulosal cells from large preovulatory follicles were exposed to greater concentrations of endogenous LH for several hours and thereafter to supplemented LH during in vitro incubation. Second, removing granulosal cells in this particular follicular developmental stage and culturing them in a serum free culture medium may have triggered their luteinization; as judged by greater concentrations of P_4 than E_2 in the culture media. Third, as occurs during luteolysis [43], END1 may act in conjunction with other locally produced ovarian mediators regulating follicular steroid production, and therefore, the absence of these factors in the culture media may have allowed luteinization of granulosal cells. Apparently, a more appropriate culture system would be necessary to test the role of END1 in steroid production by granulosal cells in large preovulatory follicles in sheep. Alternatively, collection of preovulatory follicles earlier, and incorporation of FSH and serum in culture media, may allow elucidation of the role of END1 in modulating steroid production by granulosal cells prior to ovulation in ewes. In earlier in vitro studies, FSH-simulated P_4 production by granulosal cells collected from small- and medium-sized swine follicles was inhibited by END1 [16, 18, 37]. However, END1 also reduced production of LH-stimulated P_4 by granulosal cells isolated from medium-sized follicles in pigs [30].

CONCLUSION

Granulosal cells from preovulatory follicles with greater concentration of E_2 in FF had more steroidogenic capacity, as judged by greater accumulation of E_2 and P_4 after 24 h of incubation culture. Under the experimental conditions of this study, END1 did not affect LH-stimulated steroid production by granulosal cells from preovulatory follicles in sheep, and therefore the proposed hypothesis was not supported. Collection of younger preovulatory follicles and incorporation of FSH and serum in the culture medium may allow clarify whether END1 has a role in follicular function in the sheep.

ACKNOWLEDGMENTS

The current study was supported by Hatch Multistate Project NE1027 (WV 476) and Agriculture and Food Research

Competitive Grant no. 2010-65203-20660 from USDA National Institute of Food and Agriculture (JAF).

BIBLIOGRAPHIC REFERENCES

[1] ACOSTA, T.J.; MIYAMOTO, A.; OZAWA, T.; WIJAYAGUNAWARDANE, M.P.B.; SATO, K. Local release of steroids hormones, prostaglandin E2, and endothelin-1 from bovine mature follicle in vitro: effects of luteinizing hormone, endothelin-1, and cytokines. **Biol. Reprod.** 59:437-443. 1998.

[2] ACOSTA, T.J.; BERISHA, B.; OZAWA, T.; SATO, K.; SCHAMS, D.; MIYAMOTO, A. Evidence for a local endothelin-angiotensin-atrial natriuretic peptide system in bovine mature follicles in vitro: effects on steroid hormones and prostaglandin secretion. **Biol. Reprod.** 61:1419-1425. 1999.

[3] AMSTERDAM, A.; BERKOWITZ, A.; NIMROD, A.; KOHEN, F. Aggregation of luteinizing hormone receptors in granulosa cells: a possible mechanism of desensitization to the hormone. **Proc. Natl. Acad. Sci.** 77:3440-4. 1980.

[4] BAO, B.; GARVERICK, H.A.; SMITH, G.W.; SMITH, M.F.; SALFEN, B.E.; YOUNGQUIST, R.S. Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. **Biol. Reprod.** 56:1158-1168. 1997.

[5] BAO, B.; GARVERICK, H.A.; SMITH, G.W.; SMITH, M.F.; SALFEN, B.E.; YOUNGQUIST, R.S. Expression of messenger ribonucleic acid (mRNA) encoding 3beta-hydroxysteroid dehydrogenase delta4,delta5 isomerase (3beta-HSD) during recruitment and selection of bovine ovarian follicles: identification of dominant follicles by expression of 3beta-HSD mRNA within the granulosa cell layer. **Biol. Reprod.** 56:1466-1473. 1997.

[6] BARRETT, D.M.; BARTLEWSKI, P.M.; DUGGAVATHI, R.; DAVIES, K.L., RAWLINGS, N.C. Suppression of follicle wave emergence in cyclic ewes by supraphysiologic concentrations of estradiol-17beta and induction with a physiologic dose of exogenous ovine follicle-stimulating hormone. **Biol. Reprod.** 75:633-641. 2006.

[7] BARTLEWSKI, P.M.; BEARD, A.P.; COOK, S.J.; CHANDOLIA, R.K.; HONARAMOOZ, A.; RAWLINGS, N.C. Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. **J. Reprod. Fertil.** 115:111-1124. 1999.

[8] BEG, M.A.; BERGFELT, D.R.; KOT, K.; WILTBANK, M.C.; GINTHER, O.J. Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. **Biol. Reprod.** 64:432-441. 2001.

[9] BRADFORD, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.** 72:248-254. 1976.

[10] CAMPBELL, B.K.; SOUZA, C.J.; SKINNER, A.J.; WEBB, R.; BAIRD, D.T. Enhanced response of granulosa and theca cells from sheep carriers of the FecB mutation in vitro to gonadotropins and bone morphogenic protein-2, -4, and -6. **Endocrinol.** 147:1608-1620. 2006.

[11] DENKOVA, R.; BOURNEVA, V.; BALEVA, K.; YANEVA, E.; NIKOLOV, B.; CHRISTOV, I.; IVANOV, I. Modulation of steroidogenesis in human ovarian granulosa cells during aging. **Endocr. Regul.** 34:157-160. 2000.

[12] DENKOVA, R.; BOURNEVA, V.; YANEVA, E.; BALEVA, K.; NIKOLOV, B.; IVANOV, I.; SIMENOV, K.; TIMEVA, T. Potential role of nitric oxide in endothelin-1 provoked inhibition of progesterone secretion by isolated ovarian granulosa cells. **Endocr. Regul.** 36:19-22. 2002.

[13] DOERR, M.D.; GORAVANAHALLY, M.P.; RHINEHART, J.D.; INSKEEP, E.K.; FLORES, J.A. Effects of endothelin receptor type-A and type-B antagonists on prostaglandin F2alpha-induced luteolysis of the sheep corpus luteum. **Biol. Reprod.** 78:688-696. 2008.

[14] DUGGAVATHI, R.; BARTLEWSKI, P.M.; BARRETT, D.M.; GRATTON, C.; BAGU, E.T., RAWLINGS, N.C. Patterns of antral follicular wave dynamics and accompanying endocrine changes in cyclic and seasonally anestrous ewes treated with exogenous ovine follicle-stimulating hormone during the inter-wave interval. **Biol. Reprod.** 70:821-827. 2004.

[15] EKSTROM, R.C.; HUNZICKER-DUNN, M. Guanosine triphosphate fulfills a complete and specific nucleotide requirement for luteinizing hormone-induced desensitization of pig ovarian adenylyl cyclase. **Endocrinol.** 125:2470-2474. 1989.

[16] FLORES, J.A.; QUYYUMI, S.; LEONG, D.A.; VALDHUIS, J.D. Actions of endothelin-1 on swine ovarian (granulosa) cells. **Endocrinol.** 131:1350-1358. 1992.

[17] FLORES, J.A.; WINTERS, T.A.; KNIGHT, J.W.; VELDHUIS, J.D. Nature of endothelin binding in the porcine ovary. **Endocrinol.** 136:5014-5019. 1995.

[18] FLORES, J.A.; GARMEY, J.C.; LAHAV, M.; VELDHUIS, J.D. Mechanisms underlaying endothelin's inhibition of FSH-stimulated progesterone production by ovarian granulosa cells. **Mol. Cel. Endocrinol.** 156:169-178. 1999.

[19] FLORES, J. Gene expression of endothelin in the porcine ovary: follicular development. **Biol. Reprod.** 63:1377-1382. 2000.

[20] FORTUNE, J.E; HANSEL, W. Concentrations of steroids and gonadotropins in follicular fluid from normal heifers

and heifers primed for superovulation. **Biol. Reprod.** 32:1069-1079. 1985.

[21] GHINEA, N.; VU HAI, M.T.; GROYER-PICARD, M.T.; HOULLIER, A.; SCHOËVAËRT, D.; MILGROM, E. Pathways of internalization of the hCG/LH receptor: immunoelectron microscopic studies in Leydig cells and transfected L-cells. **J. Cell. Biol.** 118:1347-1358. 1992.

[22] GINTHER, O.J.; KOT, K.; WILTBANK, M.C. Associations between emergence of follicular waves and fluctuations in FSH concentrations during the oestrous cycle in ewes. **Theriogenol.** 43:689-703. 1995.

[23] GIRSH, E.; MILVAE, R.A.; WANG, W.; MEIDAN, R. Effect of endothelin-1 on bovine luteal cell function: role in prostaglandin $PGF_{2\alpha}$-induced antisteroidogenic action. **Endocrinol.** 137:1306-1312. 1996.

[24] GONZALEZ-BULNES, A.; SANTIAGO-MORENO, J.; GARCIA-GARCIA, R.M.; DEL CAMPO, A.; GÓMEZ-BRUNET, A.; LOPEZ-SEBASTIAN, A. Origin of the preovulatory follicle in Mouflon sheep (Ovis gmelini musimon) and effect on growth of remaining follicles during the follicular phase of oestrous cycle. **Anim. Reprod. Sci.** 65:265-272. 2001.

[25] GOODMAN, R.L.; COOLEN, L.M.; ANDERSON, G.M.; HARDY, S.L.; VALENT, M.; CONNORS, J.M.; FITZGERALD, M.E.; LEHMAN, M.N. Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep. **Endocrinol.** 145:2959-2967. 2004.

[26] GUDERMANN, T.; BIRNBAUMER, M.; BIRNBAUMER, L. Homologous desensitization of the murine luteinizing hormone receptor expressed in L cells. **Mol. Cell. Endocrinol.** 110:125-135. 1995.

[27] HAWK, H.W. Uterine motility and sperm transport in the estrous ewe after prostaglandin induced regression of corpora lutea. **J. Anim. Sci.** 37:1380-1385. 1973.

[28] IRELAND, J.J.; ROCHE, J.F. Development of antral follicles in cattle after prostaglandin-induced luteolysis: changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. **Endocrinol.** 111:2077-2086. 1982.

[29] IRELAND, J.J.; ROCHE, J.F. Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. **Endocrinol.** 112:150-156. 1983.

[30] IWAI, M.; HASEGAWA, M.; TAII, S.; SAGAWA, N.; NAKAO, K.; IMURA, H.; NAKANISHI, S.; MORI, T. Endothelins inhibit luteinization of cultured porcine granulosa cells. **Endocrinol.** 129:1909-1914. 1991.

[31] IWAI, M.; HORI, S.; SHIGEMOTO, R.; KAMZAKI, H.; MORI, T.; NAKANISHI, S. Localization of endothelin receptor messenger ribonucleic acid in the rat ovary and fallopian tube by in situ hybridization. **Biol. Reprod.** 49:675-680. 1993.

[32] JIMENEZ-KRASSEL, F.; IRELAND, J.J. Development and validation of a short-term, serum-free culture system for bovine granulosa cells: evaluation of the effects of somatotropin and growth hormone-releasing factor on estradiol production. **J. Dairy. Sci.** 85:68-78. 2002.

[33] JIMENEZ-KRASSEL, F.; WINN, M.E.; BURNS, D.; IRELAND, J.L.H.; IRELAND, J.J. Evidence for a negative intrafollicular role for inhibin in regulation of estradiol production by granulosa cells. **Endocrinol.** 144:1876-1886. 2003.

[34] JO, M.; KOMAR, C.M.; FORTUNE, J.E. Gonadotropin surge induces two separate increases in messenger RNA for progesterone receptor in bovine preovulatory follicles. **Biol. Reprod.** 67:1981-1988. 2002.

[35] JUENGEL, J.L.; HUDSON, N.L.; HEATH, D.A.; SMITH, P.; READER, K.L.; LAWRENCE, S.B.; O'CONNELL, A.R.; LAITINEN, M.P.; CRANFIELD, M.; GROOME, N.P.; RITVOS, O.; MCNATTY, K.P. Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. **Biol. Reprod.** 67:1777-1789. 2002.

[36] KAMADA, S.; KUBOTA, T.; HIRATA, Y.; TAGUCHI, M.; EGUCHI, S.; MARUMO, F.; ASO, T. Direct effect of endothelin-1 on granulosa cells of the porcine ovary. **J. Endocrinol.** 134:59-66. 1992.

[37] KAMADA, S.; KUBOTA, T.; HIRATA, Y.; IMAI, T.; TAGUCHI, M.; MARUMO, F.; ASO, T. Endothelin-1 is an autocrine/paracrine regulator of porcine granulosa cells. **J. Endocrinol. Invest.** 16:425-431. 1993.

[38] KARSCH, F.J.; FOSTER, D.L.; BITTMAN, E.L.; GOODMAN, R.L. A role for estradiol in enhancing luteinizing hormone pulse frequency during the follicular phase of the estrous cycle of sheep. **Endocrinol.** 113:1333-1339. 1983.

[39] LOPEZ-SEBASTIAN, A.; GONZALEZ-BULNES, A.; SANTIAGO-MORENO, J.; GOMEZ-BRUNET, A.; TOWNSEND, E.C.; INSKEEP, E.K. Patterns of follicular development during the estrous cycle in monovular Merino del Pais ewes. **Anim. Reprod. Sci.** 48:279-291. 1997.

[40] MURDOCH, W.J.; DUNN, T.G. Alterations in follicular steroid hormones during the preovulatory period in the ewe. **Biol. Reprod.** 27:300-307. 1982.

[41] RAO, M.C.; RICHARDS, J.S.; MIDGLEY, A.R.; REICHERT, L.E. Regulation of gonadotropin receptors by luteinizing hormone in granulosa cells. **Endocrinol.** 101:512-523. 1977.

[42] SHEFFEL, C.E.; PRATT, B.R.; FERRELL, W.L.; INSKEEP, E.K. Induced corpora lutea in the postpartum beef cow. II. Effects of treatment with progestogen and gonadotropins. **J. Anim. Sci.** 54:830-836. 1982.

[43] SKARZYNSKI, D.J.; FERREIRA-DIAS, G.; OKUDA, K. Regulation of luteal function and corpus luteum regression in cows: hormonal control, immune mechanisms and intercellular communication. **Reprod. Dom. Anim.** 43(Suppl 2):57-65. 2008.

[44] SOUZA, C.J.; CAMPBELL, B.K.; BAIRD, D.T. Follicular dynamics and ovarian steroid secretion in sheep during the follicular and early luteal phases of the estrous cycle. **Biol. Reprod.** 56:483-488. 1997.

[45] SPICER, L.J.; CHAMBERLAIN, C.S.; MACIEL, S.M. Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. **Domest. Anim. Endocrinol.** 22:237-254. 2002.

[46] SPICER, L.J. Effects of estradiol on bovine thecal cell function in vitro: dependence on insulin and gonadotropins. **J. Dairy Sci.** 88:2412-2421. 2005.

[47] SPICER, L.J.; AAD, P.Y.; ALLEN, D.T.; MAZERBOURG, S.; PAYNE, A.H.; HSUEH, A.J. Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: influence of follicle size on responses to GDF9. **Biol. Reprod.** 78:243-253. 2008.

[48] STATISTICAL ANALYSIS SYSTEMS INSTITUTE (SAS). User's Guide. University North of Caroline, USA. 1999.

[49] TEDESCHI, C.; HAZUM, E.; KOKIA, E.; RICCIARELLI, E.; ADASHI, E.Y.; PAYNE, D.W. Endothelin-1 as luteinization inhibitor: inhibition of rat granulosa cell progesterone accumulation via selective modulation of key steroidogenic steps affecting both progesterone formation and degradation. **Endocrinol.** 131:2476-2478. 1992.

[50] TEDESCHI, C.; LOHMAN, C.; HAZUM, E.; ITTOOP, O.; BEN-SHLOMO, I.; RESNICK, C.E.; PAYNE, D.W.; ADASHI, E.Y. Rat ovarian granulosa cell as a site of endothelin reception and action: attenuation of gonadotropin-stimulated steroidogenesis via perturbation of the A-kinase signaling pathway. **Biol. Reprod**. 51:1058-1065. 1994.

[51] TIAN, X.C.; BERNDTSON, A.K.; FORTUNE, J.E. Changes in levels of messenger ribonucleic acid for cytochrome P450 side-chain cleavage and 3 beta-hydroxysteroid dehydrogenase during prostaglandin F2 alpha-induced luteolysis in cattle. **Biol. Reprod.** 50:349-356. 1994.

[52] TIAN, X.C.; BERNDTSON, A.K.; FORTUNE, J.E. Differentiation of bovine preovulatory follicles during the follicular phase is associated with increases in messenger ribonucleic acid for cytochrome P450 side chain cleavage, 3 beta-hydroxysteroid dehydrogenase, and P450 17 alpha-hydroxylase, but not P450 aromatase. **Endocrinol.** 136:5102-5110. 1995.

[53] TORTONESE, D.J.; LEWIS, P.E.; PAPKOFF, H.; INSKEEP, E.K. Roles of the dominant follicle and the pattern of oestradiol in induction of preovulatory surges of LH and FSH in prepubertal heifers by pulsatile low doses of LH. **J. Reprod. Fertil.** 90:127-1235. 1990.

[54] VOSS, A.K.; FORTUNE, J.E. Levels of messenger ribonucleic acid for cytochrome P450 17 alpha-hydroxylase and P450 aromatase in preovulatory bovine follicles decrease after the luteinizing hormone surge. **Endocrinol.** 132:2239-2245. 1993.

[55] XU, Z.; GARVERICK, H.A.; SMITH, G.W.; SMITH, M.F.; HAMILTON, S.A.; YOUNGQUIST, R.S. Expression of messenger ribonucleic acid encoding cytochrome P450 side-chain cleavage, cytochrome p450 17 alpha-hydroxylase, and cytochrome P450 aromatase in bovine follicles during the first follicular wave. **Endocrinol.** 136:981-989. 1995.

[56] YANAGISAWA, M.; KURIHARA, H.; KIMURA, S.; TOMOBE, Y.; KOBAYASHI, M.; MITSUI, Y.; YAZAKI, Y.; GOTO, K.; MASAKI, T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. **Nature.** 332:411-415. 1988.

[57] ZOR, U.; LAMPRECHT, S.A.; MISULOVIN, Z.; KOCH, Y.; LINDNER, H.R. Refractoriness of ovarian adenylate cyclase to continued hormonal stimulation. **Biochim. Biophys. Acta.** 428:761-765. 1976.