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Effects of Endothelin Receptor Type-A and Type-B Antagonists on Prostaglandin F_{2alpha}-Induced Luteolysis of the Sheep Corpus Luteum[1]

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ABSTRACT

Three experiments were designed to examine the mechanisms that govern prostaglandin (PGF_{2alpha})-induced regression of the sheep corpus luteum. Evidence is presented supporting the involvement of endothelin 1 (EDN1) in PGF_{2alpha}-induced luteolysis. Experiment 1 measured effects of PGF_{2alpha} when actions of EDN1 were blocked by sustained administration of a type-A endothelin (EDNRA) or type-B endothelin (EDNRB) antagonist in vivo. Experiment 2 examined antisteroidogenic actions of PGF_{2alpha} and EDN1 in the presence of an EDNRA or EDNRB antagonist in Day-8 luteal minces. In experiment 3, luteal cellular expression of EDNRA and EDNRB was determined immunohistochemically. Relative gene expression of *EDNRA* and *EDNRB* receptors was examined by real-time RT-PCR in Day-8 sheep corpora lutea. EDNRA, but not EDNRB, participated in antisteroidogenic actions of EDN1. During the first 12 h after PGF_{2alpha}-induced luteolysis, EDNRA antagonist did not prevent a decline in serum progesterone concentrations. Early actions of PGF_{2alpha} are either direct or mediated by something other than EDN1. However, beyond 12 h after PGF_{2alpha}, serum progesterone concentrations increased in EDNRA antagonist-treated animals until they were the same as saline-treated controls, whereas an EDNRB antagonist had no effect in vivo or in vitro. The EDNRA antagonist negated the antisteroidogenic actions of EDN1 but only partially abolished the actions of PGF_{2alpha} in vitro. In contrast, the EDNRB antagonist was ineffective in abolishing antisteroidogenic actions of EDN1 and PGF_{2alpha}. Whereas real-time RT-PCR demonstrated high expression of *EDNRA* and low expression of *EDNRB*, immunohistochemically, only EDNRA was located in small steroidogenic, endothelial, and smooth muscle cells. In summary, studies in ovine corpora lutea provided strong evidence that: 1) EDNRA, but not EDNRB, mediates antisteroidogenic actions of EDN1, 2) actions of PGF_{2alpha} are both independent of and dependent upon mediation by EDN1, and 3) small steroidogenic cells are targets for antisteroidogenic effects of EDN1. Furthermore, the results from experiment 1 suggest that the intermediary role of EDN1 may be more important in later stages of luteal regression.

corpus luteum, corpus luteum function, ovary, progesterone, steroid hormones

INTRODUCTION

Although the ability of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to regress the corpus luteum (CL) is well documented, if $PGF_{2\alpha}$ is administered outside of a developmental-specific window, it can, in a species-dependent manner, have no effect or stimulate luteal progesterone production [1, 2]. The wide range of responses elicited in vivo and in vitro by $PGF_{2\alpha}$ has led to the suggestion that the $PGF_{2\alpha}$ -induced reduction in progesterone is not due to direct actions on luteal steroidogenic cells but to indirect actions through other luteal cells via paracrine signaling molecules [3]. Although there is no doubt that intermediary paracrine cellular interactions are important during luteal regression [4–8], it is clear that there are direct actions of $PGF_{2\alpha}$ on its target luteal steroidogenic cells, including inhibition of luteal progesterone production [9].

A further complication in defining direct/indirect actions of $PGF_{2\alpha}$ is the fact that species might differ with regard to expression of $PGF_{2\alpha}$ receptors in luteal cells. In the ewe, the 7-transmembrane, high-affinity receptor for $PGF_{2\alpha}$ is expressed in large steroidogenic cells of the CL [10]. However, in cows, mRNA encoding $PGF_{2\alpha}$ receptors have been identified in both small and large steroidogenic cells and endothelial luteal cells [11]. Furthermore, $PGF_{2\alpha}$ induced increases in concentrations of intracellular calcium in both small and large steroidogenic cells and in luteal endothelial cells [9]. Expression of $PGF_{2\alpha}$ receptors and $PGF_{2\alpha}$-induced cellular responses certainly indicate direct actions of $PGF_{2\alpha}$ on luteal steroidogenic cells; nevertheless these observations do not ensure that luteolytic actions of $PGF_{2\alpha}$ are all direct.

There is evidence that luteal endothelial cells and their secretory product, endothelin 1 (EDN1), play an important role in the antisteroidogenic actions of $PGF_{2\alpha}$ [3–5, 12, 13]. EDN1 is a 21-amino acid peptide produced by endothelial cells and is a member of a family of structurally related peptides that includes EDN2 and EDN3 [14]. Two classes of receptors named type A (EDNRA) and B (EDNRB) mediate the actions of the different members of the EDN family [15]. Whereas EDNRB shows equal affinity for all three EDN peptides, EDNRA shows greatest affinity for EDN1. In the bovine ovary, EDNRA has been demonstrated to be the dominant endothelin receptor [15]. Expression of luteal EDNRB is low, and its role in the antisteroidogenic action of EDN1 is not known. The endothelin receptor types expressed in cells of the ovine CL have not been examined.

Although known to be a potent vasoconstrictor, EDN1 altered steroidogenesis in a variety of tissues [16–18]. Studies

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TABLE 1. Procedures for sheep handling and experimentation.

Day of estrous cycle	Procedure(s) performed
Day 0	Behavioral estrus (standing heat) observed. Ewes fed ad libitum.
Day 6	Ewes taken off feed, transported to controlled housing facility.
Day 8	Surgical implantation of minipump, first blood sample taken (−48 h from injection of $PGF_{2\alpha}$). Ad libitum feeding resumed.
Day 9	Second blood sample taken 24 h after surgery (−24 h from injection of $PGF_{2\alpha}$).
Day 10	Injection of $PGF_{2\alpha}$ and blood sampled at 8 am, followed by blood draws at 0800 h, 0900 h, 1000 h, 1200 h, 1400 h, 1600 h, 1800 h, and 2000 h.
Day 11	Blood sampled at 0800 h (24 h) and 2000 h (36 h).
Day 12	Blood sampled at 0800 h (48 h); ewes were subsequently killed so that treated CL could be removed.

have demonstrated antisteroidogenic actions of EDN1 in vitro and in vivo. It reduced progesterone production by the CL in vivo [6] and both basal and LH-stimulated progesterone in dispersed ovine luteal cells [4, 5]. This effect of EDN1 on ovine luteal cells was reduced by preincubation with a selective EDNRA antagonist [4, 5]. A mediatory role for EDN1 during luteolysis by $PGF_{2\alpha}$ is supported by two findings: 1) Gene expression of *EDN1* and its receptor, *EDNRA*, is great when CL are responsive to $PGF_{2\alpha}$ (~ Day 6 and beyond during the estrous cycle) [12, 15] and 2) Gene expression of the luteal endothelin system acquires responsiveness to $PGF_{2\alpha}$ during the late luteal phase [6, 9, 13, 19].

However, in ruminants, in vivo experiments designed to abolish luteolytic effects of $PGF_{2\alpha}$ by blocking the endothelin system have had only limited success. The luteolytic effects of $PGF_{2\alpha}$ were blocked only partially by a single direct intraluteal injection of the EDNRA antagonist BQ-123 [5]. Furthermore, antisteroidogenic actions of $PGF_{2\alpha}$ were not mitigated by direct infusion of an EDNRA antagonist into the bovine CL [20].

The manner in which EDN1 and $PGF_{2\alpha}$ interact during luteolysis remains controversial, and which cell types within the CL express the EDNRA and EDNRB remains unknown. Thus, three experiments were designed to examine the role of the endothelin system in mediating the luteolytic actions of $PGF_{2\alpha}$. In the first experiment, an endothelin receptor antagonist (EDNRA, EDNRB, or a combination of EDNRA and EDNRB) was delivered intraluteally by chronic infusion, and whether that treatment modified $PGF_{2\alpha}$-induced luteal regression was examined. The second experiment tested, in vitro, which endothelin receptor mediated the antisteroidogenic actions of EDN1 and $PGF_{2\alpha}$ in Day-8 luteal minces. A third experiment utilized immunohistochemistry to examine the luteal cell types expressing EDNRA and EDNRB and real-time RT-PCR to determine relative gene expression of *EDNRA* and *EDNRB*.

MATERIALS AND METHODS

Animals and Surgery

Animal use was approved by the ACUC at West Virginia University under protocol 05–1205. The timeline for in vivo experimental procedures is shown in Table 1. Ewes were observed for behavioral estrus twice daily in the presence of a vasectomized ram fitted with a marker harness, with the day of estrus designated as Day 0 [21]. Marked ewes were separated into different pens, rechecked for standing estrus, and, on Day 6, taken off feed, 48 h prior to surgery. Alzet miniosmotic pumps (model 2002; Alza, Palo Alto, CA) were loaded with either vehicle (200 μl 1:3 methanol/saline solution) or receptor antagonists in vehicle at a dosage of 2 mg. Antagonists to EDNRA used were BQ-123 Cyclo (-D-Trp-D-Asp-Pro-D-Val-Leu) (Bachem Bioscience, King of Prussia, PA), BQ-610 azepane-1-carbonyl-Leu-D-Trp(For)-D-Trp-OH (Bachem), or a combination of 1 mg each of BQ-123 and BQ-610. The antagonist to EDNRB, BQ-788 (N-cis-2,6-dimethylpiperidinocarbonyl)-D-Nle-OH (Bachem) was used alone or in combination with BQ-610 at 1 mg each (2 mg total). The Alzet miniosmotic pumps used were designed to deliver 0.52 μl/h for 14 days, and each pump was kept overnight in saline at 37°C on the day prior to surgery.

Initial sedation was induced with 0.3 mg/kg of diazepam (Valium, 5 mg/mL; Roche Pharmaceuticals, Nutley, NJ) i.v. followed by anesthesia with 0.14 mg/kg of ketamine HCL (Vetamine, 100 mg/ml; Mallinckrodt Veterinary, Mundelein, IL) i.v. Anesthesia was maintained with a closed-circuit system of halothane (Halocarbon Laboratories, Riveredge, NJ), oxygen (2.0 L/min) and nitrous oxide gas (1.0 L/min). During midventral laparotomy, the ovaries were examined and, if multiple CL were present, one was selected for implantation of a catheter for delivery of the antagonist. Additional CL were enucleated, and an absorbable gelatin sponge (Gelfoam; Pfizer, New York, NY) sutured in the surgical area was used as a hemostatic device. For this, the surface ovarian tissue was carefully sutured using a nonabsorbable nylon monofilament (4.0 Prolene; Ethicon, Somerville, NJ). The minipump was sutured to the pedicle near the attachment of the ovary, and a vinyl catheter tubing (Cat. no. 007760, DURECT, Cupertino, CA) connected to the pump was inserted into the CL through an incision made with a blunt probe. The catheter was stabilized within the CL by being sutured to the connective tissue capsule and the ovarian tunica albuginea, using the same nylon monofilament (Ethicon).

Following surgery, ewes received 10 mL of penicillin i.m. On Day 10, an i.m. injection of 25 mg $PGF_{2\alpha}$ analog (Lutalyse; Pfizer Animal Health, New York, NY) was given to induce luteolysis, and jugular vein blood was drawn at 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, and 48 h. Following the blood collection at 48 h, the sheep were killed, proper location of the pump and tubing was confirmed, and the CL were collected. Following removal of connective tissue, CL were weighed, sectioned into fragments, frozen, and stored in liquid nitrogen. Individual fragments were weighed, homogenized in PBS (2 mL), centrifuged, and the supernatant kept for progesterone radioimmunoassay. Blood samples were stored at 4°C and processed within 12 h of collection by centrifugation at 3000 rpm, followed by removal and storage of serum at −20°C for later RIA.

In Vitro Experiments

Corpora lutea that were collected during midventral laparotomy were utilized for in vitro experiments. Connective tissue surrounding the CL was removed, and the CL were weighed and minced into roughly 1-mm^3 pieces in cold (4°C) saline. Corpora lutea minces were distributed equally into weighed 13 × 100 mm test tubes and exposed to their respective treatments (see Figs. 3 and 4). All treatment solutions were prepared in medium 199 (containing 0.1% BSA). Stock $PGF_{2\alpha}$ (Cayman Chemical, Ann Arbor, MI) was prepared in dimethylsulfoxide (Pierce, Rockford, IL) and was diluted in medium 199 and used at a final concentration of 1 μg/ml as in previous studies from our laboratory using bovine CL [9]. Luteinizing hormone (NIADDK-oLH-25 AFP 5551B) was used at a concentration of 150 ng/ml, and EDN1 (Bachem) was used at a concentration of 1000 nM/ml. The concentration of LH used in this experiment increased the content of progesterone in media from luteal tissue isolated from Day-8 ovine CL [5]. EDN1 was used at a concentration experimentally determined to inhibit LH-stimulated progesterone content in media by Day-8 luteal minces in preliminary work. EDNRA antagonist BQ-610 or EDNRB antagonist BQ-788 was used at concentrations of 1, 10, 100, or 1000 nM.

Tissue fragments in 1 ml of their respective treatments were incubated for 4 h at 37°C in a water shaker (New Brunswick Scientific, Edison, NJ) at 150 rpm. Following incubation, media were removed and frozen at −20°C for later RIA. The experiment used Day-8 CL from four different sheep, and data for progesterone were standardized by tissue weight/tube.

Immunohistochemistry

The protocol has been validated and described [22]. Briefly, 8-μm thick sections were prepared from Day-12 frozen ovine CL from three different sheep,

TABLE 2. List of primers used for RT-PCR.

Gene	GenBank accession no.	Sequence[a]	Expected size (bp)
EDNRA	BT025379	F: GATAACCCTGAAAGCTACAGCA R: TGTGGGCAATAGTTGTGCAT	154
EDNRB	NM_174309	F: GCAGGATTTTGAAGCTCACTC R: TTTTGCTCACCAAATACAGAGC	150
Beta actin	BC102948	F: GACATCCGCAAGGACCTCTA R: ACGGAGTACTTGCGCTCAG	100

[a] F, Forward; R, reverse.

using a Richard-Allan Scientific cryostat (Series HM505 E; Fischer Scientific, Pittsburgh, PA). Ovine tissues were collected from the aorta, lungs, and uterus and used as positive controls. The tissue sections were processed for immunohistochemistry according to a previously described protocol [22]. A 5% solution of normal goat serum (NGS; Sternberger Monoclonals, Baltimore, MD) prepared in tris-buffered saline (TBS) was used to reduce nonspecific binding. Sections were incubated with either the EDNRA antibody (Chemicon International, Billerica, MA) at a dilution of 1:20 (vol/vol) or the EDNRB antibody (Chemicon International) at a dilution of 1:20 (vol/vol). Secondary antibody, anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) was used at a dilution of 1:200 (vol/vol) in 1% NGS-TBS. Tissue sections were incubated with rabbit peroxidase-antiperoxidase complex (Sternberger Monoclonals) at a dilution of 1:200 (vol/vol) in 1% NGS-TBS at room temperature for 1 h. Each treatment was performed on consecutive sections, and slides treated with the primary antibody were run simultaneously with controls. The specificity control for the immunohistological detection was determined by 1) preincubation of the primary antibody with excess antigenic peptide (1 μg peptide/1 μg antibody prepared in 1% NGS according to manufacturer's protocol), 2) omission of primary antibody, and 3) incubation with normal rabbit serum in lieu of the primary antibody. Detection was made using the substrate, 3,3-diaminobenzidine tetrahydrochloride chromogen solution (Biomedia, Foster City, CA), prepared according to the manufacturer's instructions at room temperature for 10 min. Slides were counterstained in either methyl green or Harris hematoxylin for 30 sec and were serially dehydrated at room temperature in ethanol and finally transferred into xylene for the application of coverslips using a mounting medium (Gel/Mount; Biomedia). The slides were examined later under a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) for the presence/absence of specific brown color accumulation, indicating immunoreactivity.

Real-Time RT-PCR

For the real-time quantitative determination of gene expression of *EDNRA* and *EDNRB*, total RNA was isolated from three CL collected from three different sheep with Trizol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol. Isolated RNA was quantified using the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) using 1 μl of sample. Specific primers were designed by using Primer 3 software (copyright Whitehead Institute for Biomedical Research, http://jura.wi.mit.edu/rozen/ Steve Rozen, Helen Skaletsky. All rights reserved. http://fokker.wi.mit.edu). The primer sequences and their accession numbers are shown in Table 2. The single-step RT-PCR was carried out, and cDNA product for each gene was column purified. Ten-fold serial dilutions of cDNA for each of the genes were used as templates to generate standard curves. Total RNA samples were reverse transcribed and used as templates in an iQ5 cycler (Bio-Rad Laboratories, Hercules, CA). The 25-μl reaction mixture contained 12.5 μl SYBER green mix (Bio-Rad), 2 μl cDNA sample, 2.5 μl each sense and antisense primers (0.5 μmol), and 5.5 μl of RNase-free H_2O. The standard curves of threshold cycle (ct value) versus log starting quantity for the genes of interest were obtained. The conditions used were as follows: inactivation of RT enzyme, 95°C for 3 min; denaturation, 95°C for 30 sec; annealing, 55°C for 30 sec; and extension 72°C for 1 min with fluorescence acquisition. The melt curves were generated from 55°C to 95°C with 0.5°C increments. The melt curves were observed for the presence of single amplification product. The slope and intercept values obtained from the standard curve were used to determine the starting quantity for each gene, using a linear regression equation, and gene expression for the desired gene was normalized using *β-Actin* as the reference gene.

Radioimmunoassays for Progesterone

Progesterone in CL extracts, serum samples, and media was determined by radioimmunoassay as previously described [23]. Data were expressed as mean progesterone ± SEM. Intra- and inter-assay coefficients of variance were 10.3% and 12.52% respectively, and the range of the standard curve for progesterone was from 0.10 ng/ml to 8 ng/ml.

Statistical Analysis

Statistical analyses for differences among treatments in concentrations of progesterone in serum, luteal extracts, and media were performed using JMP 3.0 from Statistical Analysis System [24]. Data were distributed normally and are presented as mean ± SEM for each experiment. Experiments were analyzed by one-way ANOVA, and the Tukey-Kramer honestly significant difference (HSD) test was used to compare different treatments [25]. A value of $P < 0.05$ was considered to be statistically different. For the densitometry data, the means of normalized expression of *EDNRA* and *EDNRB* were analyzed by paired Student *t*-tests, and $P < 0.05$ was considered significantly different.

For immunohistochemistry, 12 slides were chosen (6 EDNRA antibody, 6 EDNRA with antigenic peptide control) from three different sheep for quantification of cell types expressing immunoreactivity (Table 3). Determination of large luteal cells and small luteal cells was based on the size and shape of the observed cells relative to one another. Determination of microvascular endothelial cells or endothelial cells of large vessels was based on the size of

TABLE 3. Percentage of ovine luteal cells showing immunoreactivity for EDNRA primary antibody treated CL slides and antigenic peptide controls.

Cell type	Total cells counted (EDNRA primary antibody)[a]	Percentage showing immunoreactivity for EDNRA primary antibody	Total cells counted (immunoreactivity for EDNRA primary antibody & antigenic peptide [control])[a]	Percentage showing immunoreactivity in antigenic peptide controls
Large luteal cells	2167	3.2% (70/2167)	1742	0%
Small luteal cells	4719	64% (3014/4719)	2506	0.2% (6/2506)
Microvascular endothelial cells	804	97% (778/804)	209	14% (30/209)
Endothelial cells of large vessels	361	95% (344/361)	158	7% (11/158)
Smooth muscle cells	85	88% (75/85)	84	0%

[a]Cell counts represent total cell numbers quantified from 12 different slides (6 EDNRA, 6 control) taken from three different sheep (n = 3) for both treatment groups.

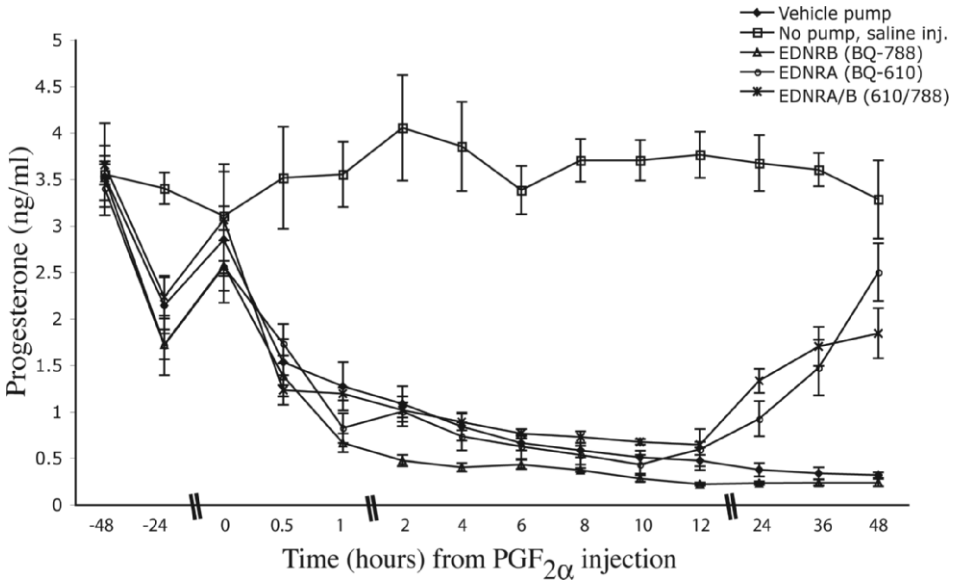

FIG. 1. The effect of a sustained administration of either BQ-610 (EDNRA antagonist), BQ-788 (EDNRB antagonist), both antagonists, or vehicle during $PGF_{2\alpha}$-induced luteolysis on the serum concentration of progesterone. Data points represent mean serum progesterone (ng/ml) for treatment groups. Values (n) for vehicle pump, no pump/saline injection (inj.), EDNRA antagonist pump, EDNRB antagonist pump, and EDNRA/EDNRB (EDNRA/B) antagonist pump were 6, 3, 10, 5, and 5, respectively. Serum progesterone concentrations for ewes treated with BQ-610 and BQ-610/BQ-788 (610/788) were greater than the vehicle control at 24, 36, and 48 h after Lutalyse injection ($P < 0.05$). BQ-610-treated ewes were not different from saline-treated controls at 48 h after injection.

the vessel structure. Endothelial cells were identified in arterial, venous, and lymphatic large vessels. Smooth muscle cells were identified in the walls of arterial vessels.

RESULTS

Experiment 1: Effect of Sustained Intraluteal Treatment with BQ-123, BQ-610, BQ-788, or Vehicle on $PGF_{2\alpha}$-Induced Luteolysis

Serum progesterone concentration. A significant reduction ($P < 0.05$) in serum progesterone concentrations was observed on the day following surgery (Fig. 1); however, there was recovery to presurgery values by the time of injection of $PGF_{2\alpha}$ on the following day. The two animals in which no CL had been removed at surgery did not show this response, indicating that reduction in serum progesterone following surgery was due to a decrease in luteal tissue.

$PGF_{2\alpha}$ induced a reduction in serum progesterone concentrations in all treated groups within 1 h after injection ($P < 0.05$). Serum progesterone concentrations in animals injected with saline remained unchanged at ~3.5 ± 0.5 ng/ml. In vehicle-treated animals, progesterone concentrations were reduced from 3.5 ± 0.3 ng/ml at the beginning of the experiment to 0.3 ng/ml ± 0.03 by 48 h. Serum concentrations of progesterone in BQ-610-treated sheep were initially 3.4 ± 0.3 ng/ml. Eight hours after the injection of $PGF_{2\alpha}$, concentrations of progesterone in the antagonist-treated group were reduced equally to that of those animals that received only $PGF_{2\alpha}$ and the pump delivering vehicle (0.5 ± 0.1 and 0.6 ± 0.1 ng/ml, respectively). However, after 12 h, serum concentrations of progesterone in the group treated with BQ-610 or the combination of BQ-610 and BQ-788 began to increase ($P < 0.05$) compared to vehicle-treated controls. By 48 h, progesterone concentrations in BQ-610 treated ewes had reached 2.5 ± 0.3 ng/ml and were not different from those in

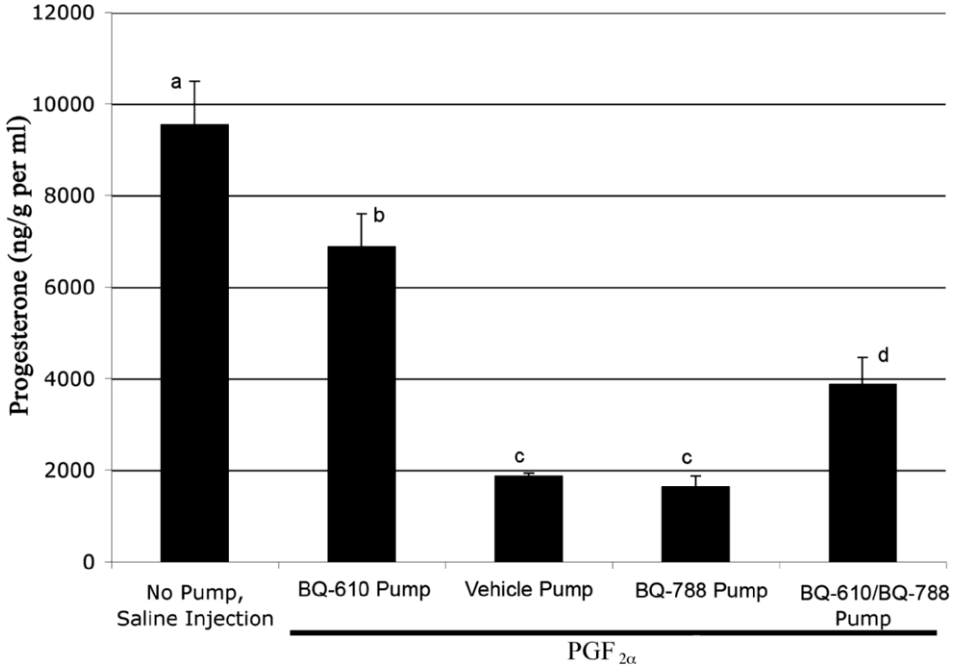

FIG. 2. The effect of a sustained administration of either BQ-610 (EDNRA antagonist), BQ-788 (EDNRB antagonist), both antagonists, or vehicle during $PGF_{2\alpha}$-induced luteolysis on the luteal progesterone content. Bars represent mean progesterone concentrations (ng/g per ml) for homogenized corpus luteum extracts. All values except vehicle pump and EDNRB, BQ-788 pump are statistically different from one another using the Tukey-Kramer HSD test ($P < 0.05$) and are denoted by different letters next to the SEM. Values (n) for vehicle pump, no pump/saline injection, EDNRA antagonist pump, EDNRB antagonist pump, and EDNRA/EDNRB antagonist pump were 6, 3, 10, 5, and 5, respectively.

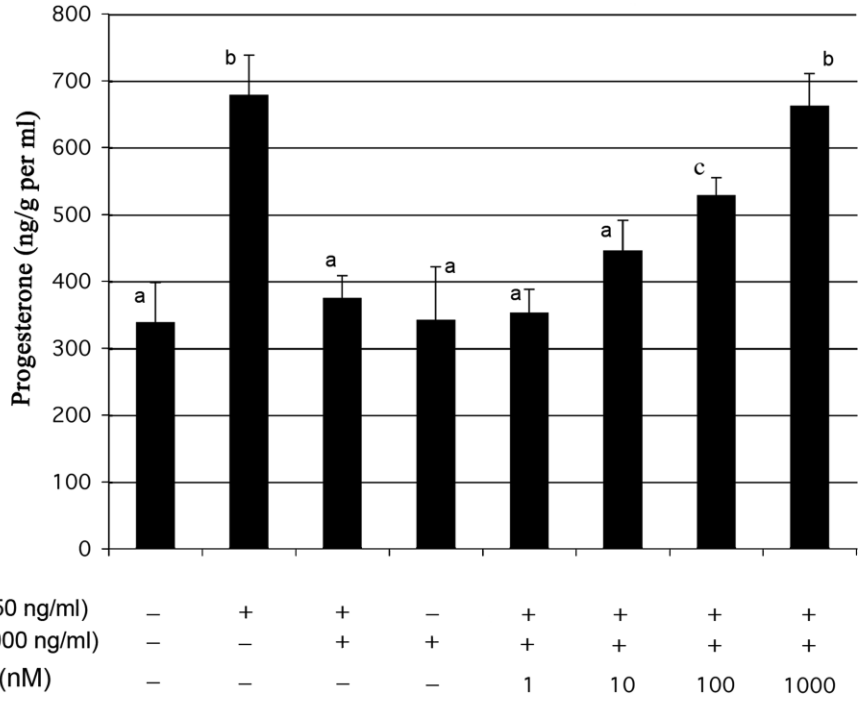

FIG. 3. Effects of LH, EDN1, and the EDNRA receptor antagonist BQ-610 in progesterone accumulation in media from incubation of Day-8 luteal minces (n = 4 CL obtained from different animals). Bars represent mean progesterone concentration in ng/g per ml of tissue. Statistical difference was determined by the Tukey-Kramer HSD test and is denoted by different letters next to the SEM.

saline-injected animals ($P = 0.065$). In contrast, the EDNRB antagonist, BQ-788, did not yield patterns different from vehicle. However, progesterone concentrations in animals that received only 1 mg of BQ-610 remained significantly lower than in saline-treated animals at 48 h. Animals treated with the BQ-123 EDNRA antagonist exhibited concentrations of progesterone that were not significantly different from those in controls that received $PGF_{2\alpha}$ without the antagonists at any time point (data not shown).

Luteal progesterone content. In vehicle-treated animals, treatment with $PGF_{2\alpha}$ significantly reduced ($P < 0.05$) luteal progesterone content by 89.9% from values observed in saline-injected controls (Fig. 2). In the experimental group receiving BQ-610, $PGF_{2\alpha}$ reduced ($P < 0.05$) luteal progesterone content by only 31%, and mean values were greater than in vehicle-treated controls ($P < 0.05$; Fig. 2). In CL treated with a combination of 1 mg each of BQ-610 and BQ-788, a reduction in luteal progesterone content of 64% was observed, compared to saline controls, and final mean values were greater than in vehicle- and BQ-788-treated groups ($P < 0.05$) but significantly lower than in groups treated with saline or 2 mg of BQ-610 ($P < 0.05$; Fig. 2).

Experiment 2: Effect of EDN1 and $PGF_{2\alpha}$ on Progesterone Production In Vitro

Luteinizing hormone increased media progesterone content above controls ($P < 0.05$), and the effect of LH was reduced to basal values by EDN1 ($P < 0.05$; Fig. 3). The effect of EDN1 was blocked in a dose-dependent manner by the addition of EDNRA antagonist BQ-610. A significant effect was observed at 100 nM BQ-610, and the addition of 1000 nM BQ-610 completely blocked the antisteroidogenic actions of EDN1 and restored values to concentrations that were not significantly different from those of LH alone. The EDNRB antagonist, BQ-

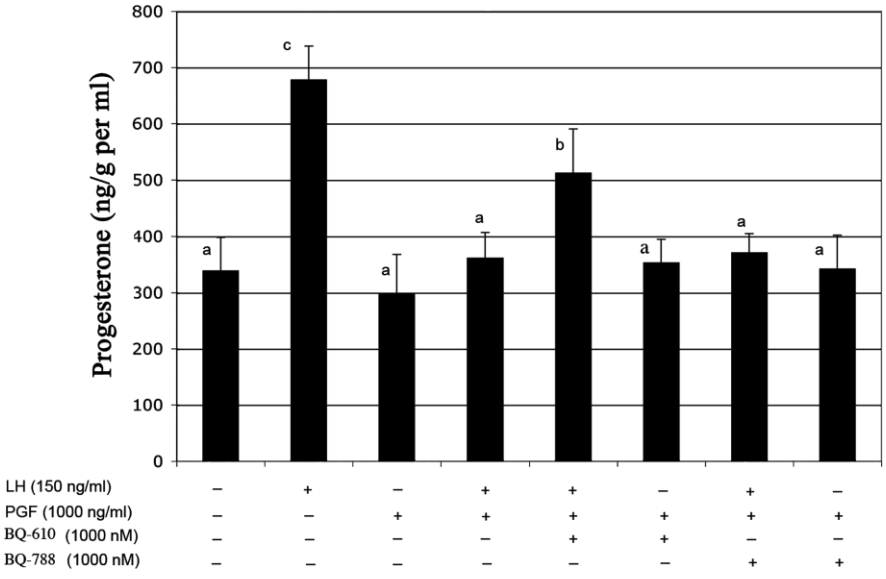

FIG. 4. Effects of $PGF_{2\alpha}$ (PGF), LH, BQ-610, and the EDNRB antagonist BQ-788 in progesterone accumulation in media from incubation of Day-8 luteal minces (n = 4 CL obtained from different animals). Bars represent mean progesterone concentration in ng/g per ml of tissue. Antagonist concentrations of BQ-610 and BQ-788 are 1000 nM. Statistical difference ($P < 0.05$) was determined by the Tukey-Kramer HSD test and is denoted by different letters next to the SEM.

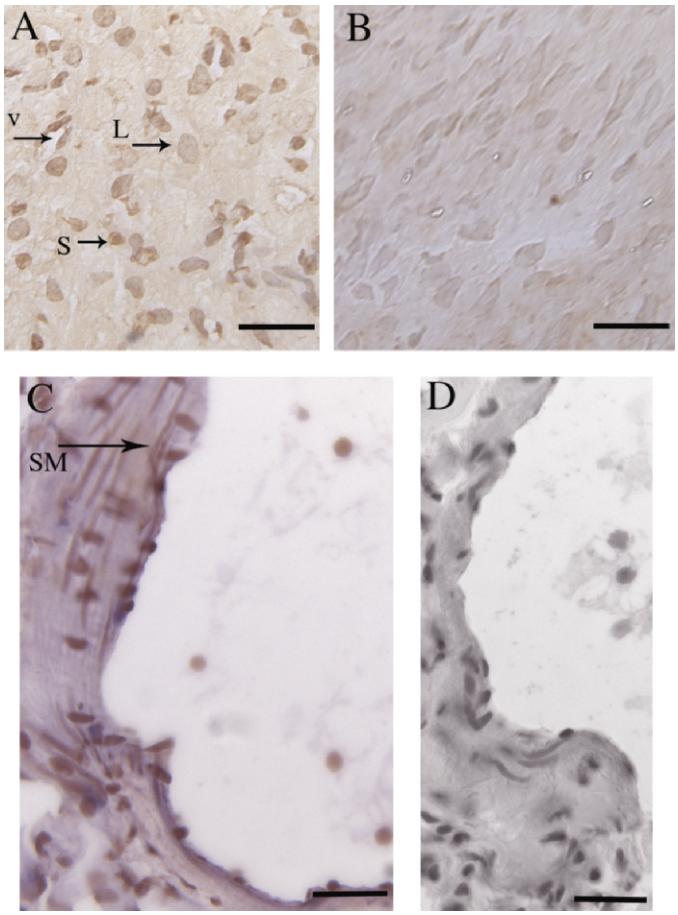

FIG. 5. Detection of EDNRA in Day-12 ovine CL (**A** and **B**) and EDNRB in lung (**C** and **D**) by immunohistochemistry. **A**) Immunoreactivity in small luteal cells (S) but not in large luteal cells (L). Only the nuclei of the large luteal cells are observable, yet due to immunoreactivity, both nuclei and cytoplasm are visible in small luteal cells. The arrow with the letter v indicates the lumen of a small blood vessel. **B**) Specificity of the assay demonstrated by the lack of immunoreactivity when the primary antibody was preincubated with the antigenic peptide. EDNRB immunoreactivity was not detected in any luteal cell (data not shown). Panel **C** demonstrates immunoreactivity (arrow) for EDNRB in smooth muscle (SM) cells of blood vessel of the lung. **D**) Specificity of the assay demonstrated by the lack of immunoreactivity when the primary antibody was preincubated with the antigenic peptide. Bar = 100 μm.

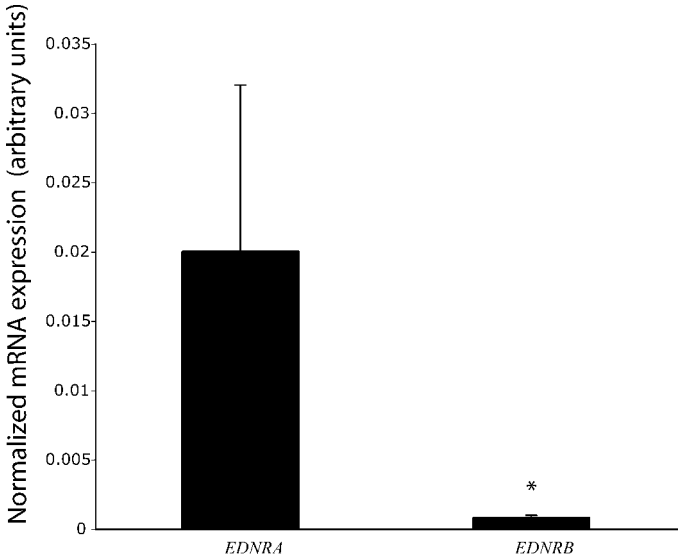

FIG. 6. The cDNAs for *EDNRA* and *EDNRB* from total RNA isolated from sheep CL (three CL collected from three different animals) were amplified in an iQ Cycler. The expression of mRNA was normalized using beta-actin as a control gene. The standard curves were generated for each gene, using a 10-fold dilution of purified PCR product, and the melt curve was analyzed for a single desired gene product. The details for the assay conditions are explained in *Materials and Methods*. The means of normalized expression values were analyzed by paired Student *t*-tests, and $P < 0.05$ was considered significantly different. Data are presented as mean ± SEM. Statistical difference ($P < 0.05$) is denoted by the symbol * next to the SEM.

788, did not block the antisteroidogenic actions of EDN1 at any concentration (data not shown). Interestingly, EDN1 did not reduce basal progesterone content in media.

Addition of $PGF_{2\alpha}$ completely blocked the steroidogenic actions of LH (Fig. 4), but it did not reduce basal progesterone content in media alone. The EDNRA antagonist reduced ($P < 0.05$), but did not eliminate, the inhibitory action of $PGF_{2\alpha}$ on LH-stimulated secretion of progesterone. In contrast, the EDNRB antagonist had no effect on the antisteroidogenic actions of $PGF_{2\alpha}$ (Fig. 4). Luteinizing hormone-stimulated progesterone content in media was not affected by either of the endothelin receptor antagonists.

Experiment 3

Immunohistochemistry. Cellular localization of EDNRA and EDNRB was examined by immunohistochemistry in CL collected from three Day-12, saline-treated sheep. Counts of populations of cells expressing immunoreactivity from each slide treated with either the EDNRA antibody or the EDNRA antibody exposed to the antigenic peptide are displayed in Table 3. Serial sections from each CL produced similar results, and representative photographs are displayed in Figure 5. Specific EDNRA immunoreactivity was detected in small luteal cells and microvascular endothelial cells but not in large luteal cells (Fig. 5A). This immunoreactivity was eliminated when the antibody was preincubated with the antigenic peptide prior to immunodetection (Fig. 5B). Immunoreactivity was also eliminated in the other two controls described in *Materials and Methods* (data not shown). EDNRB immunoreactivity was not detected in any luteal tissue; however, it was detected in positive control tissue such as lung (Fig. 5C), aorta, and uterus (data not shown).

Real-time RT-PCR. As immunoreactivity corresponding to EDNRB could not be detected in the ovine CL, expression of *EDNRB* and *EDNRA* was examined in the ovine CL. The real-time quantitative analysis of the endothelin receptors revealed that *EDNRB* was expressed but approximately at 1/10 the amount of expression for *EDNRA* (Fig. 6). The expression pattern for the endothelin receptors observed in the ovine CL was not different from the pattern observed in the bovine CL (data not shown).

DISCUSSION

The data reported here expand knowledge of several aspects of the role of EDN1 in luteal function. Two important aspects revealed in this study are: First, the data of experiment 1 strengthen the involvement of EDN1 as a mediator of $PGF_{2\alpha}$ in the process of luteal regression. A second and perhaps more important contribution revealed in experiment 1 is that early luteolytic actions of $PGF_{2\alpha}$ were independent of mediation by EDN1, whereas later antisteroidogenic actions were reversed effectively by an EDNRA antagonist. An early effect of $PGF_{2\alpha}$

in reducing serum progesterone concentrations was not abolished by endothelin receptor antagonists. This observation could mean that during this part of luteal regression, $PGF_{2\alpha}$ acts directly on luteal steroidogenic cells or via some paracrine or autocrine factor other than EDN1. The data do not allow us to discern between these two interpretations.

In a previous in vivo study, the EDNRA antagonist BQ-123 only partially blocked the actions of $PGF_{2\alpha}$ in the ewe [5]. Furthermore, this partial blockade of the antisteroidogenic effect of $PGF_{2\alpha}$ was observed to occur without delay. The discrepancies between these two studies are probably due to the fact that in the study by Hinckley and Milvae [5], a single injection of the EDNRA antagonist was used, and only 10 mg $PGF_{2\alpha}$ was used to induce luteal regression. In contrast, in the present study intraluteal delivery of the antagonist started 48 h before the injection of 25 mg $PGF_{2\alpha}$, and it was sustained throughout the duration of the experiment. The induced decline in progesterone concentrations in the report by Hinckley and Milvae was of less magnitude and took longer than in the present study. Furthermore, in the present study, BQ-123 did not reverse the luteolytic actions of $PGF_{2\alpha}$. This discrepancy in the effectiveness of BQ-123 in vivo may result from differences in source and composition of the antagonists, Cyclo (-D-Trp-D-Asp-Pro-D-Val-Leu) (BQ-123; Bachem) [current study], or Cyclo (D-Asp-Pro-D-Val-Leu-D-Trp) (BQ-123; Sigma, St. Louis, MO) as used in the study by Hinckley and Milvae [5]. Additionally, BQ-123 might be less effective in vivo when compared to BQ-610. This is supported by the observation that BQ-123 has a lower pA_2 value (7.4) [26] than BQ-610 (8.2; Bachem). The pA_2 value quantifies the action exerted by an inhibitor [26], and thus supports the hypothesis that BQ-123 is less effective at blocking the EDNRA than BQ-610. Therefore, a single injection of a less potent antagonist possibly could have resulted in an incomplete blockade of endogenous EDN1 in the study by Hinckley and Milvae [5].

Examination of luteal progesterone content yielded results similar to, but not exactly the same as, those observed in serum. Interestingly, the EDNRA antagonist did not completely restore luteal progesterone content to those of saline control values. Moreover, CL treated with a combination of both antagonists had progesterone content that was significantly lower than in those that received twice the concentration of EDNRA antagonist alone, indicating a dose-dependent response that was not observed in the data for serum progesterone. In a previous report, Gomes et al. [27] found that progesterone in jugular vein serum was not predictive of progesterone content of the nonpregnant bovine CL; therefore luteal content might be more reliable than serum progesterone concentration. Although delivery of the antagonist began 48 h prior to injection of $PGF_{2\alpha}$ and was continued for the duration of the experiment, perhaps a higher antagonist concentration could have allowed complete restoration of luteal progesterone content to values not different from those of the saline control group. However, the possibility that the observed delay was due to an incomplete blockade of endogenous EDN1 is unlikely. A more likely interpretation is that $PGF_{2\alpha}$ has early, EDN1-independent actions and later luteolytic actions that require EDN1 acting through the EDNRA. Furthermore, these results indicate that the role of EDN1 in luteal regression might be of greater significance during the later stages of luteolysis.

The current study helps to distinguish some of the reasons for the discrepancy in the relative ability of an EDNRA to reverse $PGF_{2\alpha}$-induced luteolysis [5, 20]. It was unclear whether the limited success [5] or no success at preventing $PGF_{2\alpha}$-induced reduction in serum progesterone concentrations [20] could arise from effectiveness in the method of introducing antagonists to the CL or to species differences. Although it is possible that species differences might exist between the cow and sheep, it is now clear that the method of administering the antagonist, a single intraluteal injection [5] or sustained administration [current study] alone can explain, in part, discrepancies observed in the same species. However, although a sustained administration of BQ-610 in the current study may be a more effective method of introducing antagonists to the CL in ruminants, there are effects of $PGF_{2\alpha}$ during $PGF_{2\alpha}$-induced luteal regression that cannot be prevented by blocking EDN1 actions (Figs. 1 and 3). The inability of an EDNRA antagonist to prevent the antisteroidogenic actions of $PGF_{2\alpha}$ in the cow could be due to the fact that the antagonist was administered 10 h after the $PGF_{2\alpha}$ treatment [21], a time at which the antisteroidogenic actions of $PGF_{2\alpha}$ were still EDN1-independent (Fig. 1). The existence of species differences is emphasized by the report that, in the rabbit, EDN1 alone induced luteolysis and that action was prevented by injection of an EDNRA antagonist [28]. Thus, although the importance of EDN1 in luteolysis may differ in a species-dependent manner, the time at which the antagonist is administered might be critical. Clearly, in the ewe, obligatory roles for both $PGF_{2\alpha}$ and EDN1 have been established.

The decrease in serum concentrations of progesterone after removal of additional CL (Fig. 1) was expected and did not occur in ewes with only one CL at surgery. However, by the time of treatment with $PGF_{2\alpha}$, serum progesterone had recovered to presurgery concentrations, which is a point of interest. The remaining CL may have compensated for the removal of additional CL. A similar compensatory response has been documented in response to unilateral ovariectomy during the luteal phase in the rabbit [29], rat [30], and pig [31].

The reduction in serum progesterone concentrations induced within 1 h after injection of $PGF_{2\alpha}$ agrees with previous reports using a similar protocol in the ewe [32, 33]. As reported in those studies, the dosage of $PGF_{2\alpha}$ used in this experiment reduced serum progesterone to nearly undetectable concentrations. Given that serum concentrations of progesterone at the time of injection did not differ from those prior to surgery, the reduction in serum progesterone can be attributed to $PGF_{2\alpha}$ and not to the surgical procedure.

Prior to this study, the ability to reverse luteolysis had been demonstrated with other approaches. Goyeneche et al. [34] found that androstenedione greatly reduced the decline in progesterone, the amount of luteal cell apoptosis, and the decline in luteal weight associated with luteolysis immediately following parturition in the rat. Similarly, in the cow, a high dose of tumor necrosis factor (10 μg) prolonged the estrous cycle by stimulating the production of progesterone and PGE_2 [35]. Prolongation of the luteal lifespan also has been documented in the ewe in response to chronic treatment with estradiol-17β if initiated early in the cycle [36, 37]. Additionally, chronic administration of oxytocin by osmotic minipump, jugular cannula infusion, or intraluteal injection extended the life of the CL in both the ewe [38] and the cow [39]. The findings reported here demonstrate an alternative method by which luteolysis can be effectively reversed and support the hypothesis that the ability to prevent luteolysis by chronic treatment with either agonists or antagonists may be a general feature of the ruminant CL.

It appears that it is possible to hormonally reprogram the CL and modify its lifespan to prevent its demise. If this is a general feature of the mammalian CL, it could be the basis for practical applications in the management of fertility. Given the commercial availability of an oral EDNRA antagonist (Xinlay

Atrasentan, ABT-627; Abbott Laboratories, Abbott Park, IL), blockage of the EDNRA may represent the most practical application available to prevent a decline in luteal progesterone in sheep to date.

The dose-dependent manner in which BQ-610 blocked the actions of EDN1, and the inability of BQ-788 to do the same in vitro, demonstrate that EDN1 acts through EDNRA, which is in agreement with previous findings in the sheep and cow [5, 9, 19]. Furthermore, the ability of BQ-610 to mitigate actions of $PGF_{2\alpha}$ on progesterone accumulation in vitro strongly supports previous observations in vivo and in vitro that EDN1, acting through EDNRA, can mediate some of the luteolytic effects of $PGF_{2\alpha}$ [4–6, 13]. However, the EDNRA antagonist BQ-610 did not completely block the luteolytic actions of $PGF_{2\alpha}$, so at least some actions of $PGF_{2\alpha}$ may not be mediated by EDN1, because the concentration of EDNRA antagonist used was effective in completely blocking the inhibitory effect of EDN1. However, the delayed effects of the antagonist observed in vivo could not be observed in the in vitro studies, perhaps indicating the limitations of the in vitro approach in studying the intermediary role of EDN1 during $PGF_{2\alpha}$-induced regression.

The inability of EDN1 to reduce basal progesterone production by Day-8 luteal minces is inconsistent with some previous observations in dispersed ovine [5] and bovine luteal cells [4] exposed to increasing concentrations of EDN1. However, an antisteroidogenic action of EDN1 only on LH-stimulated but not basal progesterone production would be consistent with the localization of the EDNRA in the small steroidogenic luteal cells (Fig. 5A). Large luteal cells are the primary source of basal progesterone in the ewe, with small luteal cells responding to stimulation by LH [40–42]. Therefore, it appears that the small steroidogenic cells could be the common target for the actions of LH and EDN1.

Treatment with $PGF_{2\alpha}$ did not reduce basal progesterone production in vitro. This observation is inconsistent with previous in vitro studies in the cow [9], sheep [42], and pig [43], in which $PGF_{2\alpha}$, in a dose-dependent manner, reduced basal progesterone content in media. However, there is at least one study in which $PGF_{2\alpha}$ had no effect on basal progesterone production in midphase bovine luteal slices [4]. Thus, conflicting data exist regarding the ability of $PGF_{2\alpha}$ to reduce basal progesterone content in vitro. This discrepancy may arise from differences in experimental procedures, including: species, whether cells were dispersed or associated (as in luteal minces or luteal slices), or the day of the estrous cycle from which the cells were obtained.

Although it was clear that EDNRA is expressed in the ovine CL, the immunohistological data indicate that EDNRA is located in small steroidogenic cells, and, as expected [13], in endothelial (Fig. 5A) and smooth muscle cells (data not shown). Immunoreactivity was not observed in the large steroidogenic cells. This may indicate that EDNRA is either not expressed or that the level of expression was too low to be detectable. Although this observation has to be independently confirmed, the distribution of the EDNRA on small cells could represent an additional mechanism to the one proposed by Niswender et al. [44] and Fitz et al. [45], by which the luteolytic actions of $PGF_{2\alpha}$ on LH-stimulated progesterone production could be mediated.

The presence of EDNRA on small, but not large, steroidogenic cells is inconsistent with observations in the bovine CL by Choudhary et al. [9], who reported increases in intracellular calcium concentrations in both large and small bovine luteal steroidogenic cells in response to EDN1. Similarly, Girsh et al. [4] detected high affinity and selective EDN1 binding sites in both large and small bovine luteal steroidogenic cells. Species differences between cow and sheep could account for this discrepancy. Therefore, as emphasized earlier, this observation will have to be corroborated independently by different investigators with the use of additional experimental approaches. Nevertheless, the absence, or low level of expression, of EDNRA in large luteal cells would not support the suggestion that EDN1, acting through EDNRA, has direct antisteroidogenic actions on both large and small steroidogenic cells within the sheep CL [46]. An effect of EDN1 on LH-stimulated, but not basal progesterone secretion would support the interpretation that the small luteal cells are the primary target for LH- and EDN1-mediated actions on progesterone production [40, 41].

The inability to detect EDNRB in luteal cells is clearly due to its low level of expression, because the antibody detected this receptor in positive control tissue known to have a high level of EDNRB expression (Fig. 5C). It is clear that although *EDNRB* is expressed in the sheep CL, *EDNRA* is the most abundantly expressed receptor, and the antisteroidogenic actions of EDN1 and $PGF_{2\alpha}$ in the ewe are not mediated through EDNRB. It could be that the EDNRB in the sheep CL is associated with regulation of luteal blood flow; however, the data do not allow us to discern a luteal role for EDNRB at this time.

In conclusion, these studies demonstrate that: 1) actions of $PGF_{2\alpha}$ during induced luteal regression are both independent of and dependent upon mediation by EDN1, 2) the antisteroidogenic effect of EDN1 was through the EDNRA, and 3) EDNRB did not mediate the antisteroidogenic actions of EDN1 and $PGF_{2\alpha}$. The data also indicate that the intermediary role of EDN1 in luteolysis might be more significant at later stages of luteal regression, and although additional evidence is needed, the small steroidogenic luteal cells appear to be the target for the antisteroidogenic actions of EDN1 in the ovine CL.

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