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Involvement of Endothelin-1 and Its Receptors in $PGF_{2\alpha}$-Induced Luteolysis in the Rat

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ABSTRACT The possible mediatory role of endothelin-1 (ET-1) in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced luteolysis in the rat was examined. The effect of $PGF_{2\alpha}$ was tested on day 9 of pregnancy either in vivo, by injecting cloprostenol, an analog of $PGF_{2\alpha}$ or in vitro, in isolated intact corpora lutea incubated with $PGF_{2\alpha}$. Luteolysis was confirmed by progesterone determination in the peripheral blood serum or in the culture medium, respectively. Administration of cloprostenol (.0025 mg/rat) induced within 1 hr, a significant fall (from 56.8 to 27.6 ng/ml, $P < 0.0001$) in serum progesterone concentrations that was associated with an increased expression of the mRNA to ET-1 and its protein product in rat luteal tissue. Elevated level of ET-1 were also determined at the spontaneous regression of the CL, upon parturition. Expression of the ET receptors, ETA and ETB was not affected by cloprostenol. On the other hand, this $PGF_{2\alpha}$ analog induced expression of luteal VEGF mRNA. In vitro experiments demonstrate that the LH (100 ng/ml)-induced increase in luteal progesterone secretion was reduced by $PGF_{2\alpha}$ (1 μg/ml). The inhibitory effect of $PGF_{2\alpha}$ was reversed by BQ123 (10^{-7} M), that is a selective ETA receptor antagonist. We conclude that the $PGF_{2\alpha}$-induced elevation in luteal expression of ET-1 combined with the reversal of its luteolytic effect by an ETA receptor antagonist suggest that ET-1 may take part in the $PGF_{2\alpha}$-induced luteolysis in the rat. *Mol. Reprod. Dev. 63: 71–78, 2002.*

Key Words: $PGF_{2\alpha}$; endothelin-1; corpus luteum; luteolysis; hypoxia; VEGF

INTRODUCTION

The corpus luteum (CL) that represents a later developmental stage of the post-ovulatory follicle is responsible for the formation of the secretory endometrium that is a prerequisite for implantation. This effect of the CL is mediated by progesterone. If fertilization did not occur, the functional life of the CL is terminated at the end of the estrous cycle. Alternatively, in a pregnant animal, the life span of the CL is prolonged for a period that varies among species. Regression of the CL, also known as luteolysis, is characterized by functional and structural alterations, which result in the loss of the steroidogenic capacity of the luteal cells. Luteolysis in rodents, ruminants, and primates is induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Pharriss and Wyngarden, 1969; Thorburn et al., 1972; Summers et al., 1985; Basu et al., 1987). In most nonprimate mammalian species, the luteolytic $PGF_{2\alpha}$ is produced by the endometrium (Thatcher and Chenault, 1976; McCracken et al., 1984). Luteolysis in primates, on the other hand, is controlled by local luteal $PGF_{2\alpha}$ production (Shutt et al., 1976).

Despite of the well-established luteolytic properties of $PGF_{2\alpha}$, the mechanisms involved in the induction of luteolysis are not fully defined. The effect of $PGF_{2\alpha}$ that was evident only in the presence of endothelial cells suggested that these cells of the luteal capillaries are essential for the induction of luteolysis by $PGF_{2\alpha}$ (Girsh et al., 1995). Later reports indeed demonstrated that $PGF_{2\alpha}$ rapidly elevates luteal endothelin-1 (ET-1) (Ohtani et al., 1998) and that ET-1 inhibited luteal progesterone (Girsh et al., 1996a; Miyamoto et al., 1997a). Moreover, $PGF_{2\alpha}$-induced reduction in luteal progesterone in cows (Girsh et al., 1996a) and ewes (Hinckley and Milvae, 2001) is reversed by administration of an antagonist to the endothelin receptor type ETA. These observations suggest that functional luteolysis is mediated by luteal ET-1, the product of endothelial cells.

The trigger for luteinization in mammals is provided by the pituitary luteinizing hormone (LH). However, unlike the other mammalian species that spontaneously develop a luteal phase after ovulation, rat and mice postovulatory corpora lutea require luteotrophic support in order to become functional. These unique characteristics of rodents corpora lutea provoked our interest in mechanisms involved in luteolysis in the rat. Specifically, we investigated the possible mediatory role of ET-1 in $PGF_{2\alpha}$ action on the CL in this animal model. We further raised the hypothesis that the ET-1 mediated $PGF_{2\alpha}$-induced luteolysis may involve vasoconstriction resulting in hypoxic conditions that could participate in termination of function of this tissue.

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MATERIALS AND METHODS

Chemicals, Drugs, and Reagents

The materials utilized in the present study were obtained from the following sources. Highly specific monoclonal antibodies to progesterone (P4) were generously provided by Dr. F. Kohen (The Weizmann Institute of Science, Rehovot, Israel). Rabbit antibodies to mouse immunoglobulins were purchased from Dako A/S (Glostrup, Denmark). Sheep antibodies to ETA receptor were purchased from Biogenesis (London, UK). Rabbit antibodies to ETA and to ETB receptors were purchased from Alomone labs (Jerusalem, Israel). HRP-goat anti rabbit IgG (H + L) and HRP-donkey anti sheep IgG (H + L) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Protein assay reagent was from Bio-Rad Laboratories (GmbH, Munich, Germany). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Life Science (Buckinghamshire, UK). Estrumate (cloprostenol, an analog of $PGF_{2\alpha}$) was purchased from Coopers Animal Health Ltd. (Berkhamsted, UK). DMEM and HEPES (vol/vol) nutrient mixture (DMEM/HEPES), BQ123, $PGF_{2\alpha}$, penicillin, streptomycin, neomycin, fetal bovine serum (FBS), bovine serum albumin (BSA, fraction V, protease free), and sepharose-protein A conjugated beads were purchased from Sigma Chemical Co. (St. Louis, MO). Ovine LH (oLH) was kindly provided by the National Institute of Health (NIH). ELISA for ET-1 (ET-1 cell culture kit) was purchased from Biomedica (Wienna, Austria). Primers for mRNA to ET-1, ETA, and ETB were from Genosys Biotech Ltd. (London, UK). Acrylamide and *bis*-acrylamide were purchased from Merck (Darmstadt, Germany). FUJI Medical X Ray Super RX Film was from FUJI Photo Film Co. (Tokyo, Japan). Nitrocellulose (0.2 μm pore size) was obtained from Schleicher and Schuell (Dassel, Germany). All other chemicals were reagent grade from Sigma Chemical Co. or Merck.

Animal Model

Sexually mature Wistar female rats (Harlan Biotech, Rehovot, Israel; body weight: 200–250 g, 12-week-old), showing three consecutive 4-day cycle (examined daily by vaginal cytology) were housed on a 14 hr light and 10 hr dark cycle schedule at 21°C and 55% humidity. The rats were allowed a free access to food and water. Proestrous females were caged with fertile males overnight, separated on the next morning and analyzed for the presence of sperm in their vagina. This day was considered as day 1 of pregnancy. Luteolysis was induced by a single intraperitoneal injection of 0.025 mg of cloprostenol, a $PGF_{2\alpha}$ analog, dissolved in saline and administered on day 9 of pregnancy. The efficiency of cloprostenol in inducing luteal regression was evaluated by serum P4 determination. Control rats were injected with saline. All protocols were conducted in accordance with the NIH Guides for the Care and Use of Laboratory Animals (National Research Council, National Academy of Science, Bethesda, MD).

Tissue Preparation

The rats were sacrificed on day 9 of pregnancy and their ovaries were removed surgically. The large corpora lutea of pregnancy (1.28 ± 0.13 mg/CL) were dissected. The small corpora lutea of previous cycles were not included in the experimental design. The corpora lutea were individually recovered and either processed for further incubation as described below or immediately frozen in liquid N_2 and stored at -80°C until use.

Corpora Lutea Culture

The isolated corpora lutea were washed in DMEM/HEPES and cultured in glass tubes (12 × 75 mm), one CL/tube. Corpora lutea were preincubated in DMEM/HEPES with 1% antibiotics (penicillin, streptomycin, and neomycin) and 5% FBS for 3 hr in a water shaking bath at 37°C followed by medium replacement to obtain equilibrium of the basal P4 secretion. Corpora lutea were then incubated for an additional 18 hr under the following conditions: control (no further additions), $PGF_{2\alpha}$ (1 μg/ml), the specific ETA receptor antagonist BQ123 (10^{-7} M), oLH (100 ng/ml), and their combination as indicated. Maximal secretion of P4 from rat luteal cells is obtained by a concentration of 100 ng of LH (Nelson et al., 1992). The tubes were sealed and incubation was carried out under an atmosphere of N_2:O_2 (1:1) at 37°C. At the end of the experiment the weight of each CL was monitored and the incubation medium kept frozen at -80°C for P4 determination.

Evaluation of ET-1, ETA, and ETB Receptors and VEGF Genes Expression

The expression of ET-1, ETA, and ETB genes was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted by the acid-guanidinium-phenol-chloroform method. The precipitated RNA was washed with 70% ethanol. The concentration of the extracted RNA was calculated by determination of the OD at 260 nm. The ratio of the optical density of 260–280 nm was always greater than 1.9.

Aliquots of total RNA (1 μg) were reverse-transcribed using random primers. RT reaction contained 50 U of moloney murine leukemia virus reverse transcriptase (MLV-RT), 200 μM dNTP, 6.5 mM $MgCl_2$, 20 U of RNasin, 0.5 ng oligo dT, and 1.5 × PCR buffer in a total volume of 20 μl. The reaction was performed at 37°C for 2 hr. The vials were stored at -80°C until PCR was performed. Fragments of the reverse transcribed ET-1 cDNA were amplified using a radiolabeled nucleotide ([α-^{32}P]dCTP) and pairs of specific primers. The sequence of the primers used is shown in Table 1. The cDNA amplification products for ET-1 were predicted to contain 382 base pairs (bp). PCR reactions were further performed in the same RT-test vial that finally contained 20 μM of each primer, 200 μM dNTP, 2.5 mM $MgCl_2$, 2 μCi [α-^{32}P]dCTP, 1 × PCR buffer, and 2.5 U of *Taq* polymerase to amplify a portion of the cDNA. The

TABLE 1. Sequence of the Primers Used in the Study

Gene	Bases	Primers
ET-1	Sense (340–361)	5′-TTG CTC CTG CTC CTC CTT GATG-3′
	Antisense (721–700)	5′-GGT CTT GAT GCT GTT GCT GATG-3′
ET_A	Sense (121–140)	5′-CAG TGC TAA TCT AAG CAG CC-3′
	Antisense (518–497)	5′-TGC AGA GAA ACA CTC CAA AAT C-3′
ET_B	Sense (307–326)	5′-AGG CCA CAC CAT CTC TTC TC-3′
	Antisense (727–709)	5′-AGC TTG CAC ATC TCA GCT C-3′
VEGF	Sense (324–345)	5′-CAA ACC TCA CCA AAG CCA GCA C-3′
	Antisense (528–506)	5′-CAA ATG CTT TCT CCG CTC TGA AC-3′
S-16	Sense	5′-CGT TCA CCT TGA TGA GCC CAT T-3′
	Antisense	5′-TCC AAG GGT CCG CTG CAG TC-3′

number of PCR cycles for each product was in the linear range of the amplification curve. Thirty-one cycles for ET-1 were employed after incubation of 2 min at 94°C as follows: 94°C for 1 min (denaturation); 62°C for 1 min (annealing); and 72°C for 2 min (elongation) followed by a final extension for 10 min at 72°C. The radioactive products were size-fractionated by 5% non-denaturing polyacrylamide gel electrophoresis in 0.5 × TBE buffer. Gels were dried and radioactivity determined by their exposure to X-ray film.

PCR for the ET receptors was performed with the specific primers, directed to the non-homologous areas. The cDNA amplification products were predicted to be 398 bp in length for the ETA receptor and 421 bp for the ETB receptor. The following conditions were used for the PCR reaction: 25 cycles both for ETA and for ETB were employed after incubation of 2 min at 94°C as follows: 94°C for 30 sec (denaturation); 58°C for 30 sec (annealing) and 72°C for 1 min (extension) followed by a final extension for 5 min at 72°C.

The following conditions were used for the PCR reaction for VEGF: 26 cycles were employed after incubation of 5 min at 95°C as follows: 95°C for 1 min (denaturation); 58°C for 1 min (annealing); and 72°C for 2 min (extension) followed by a final extension for 10 min at 72°C.

The identity of the PCR products was confirmed by direct DNA sequencing. Quantitation was performed by densitometric analysis of the autoradiograms normalized against ribosomal protein S-16 mRNA that served as an internal standard. Densitometric analysis was performed utilizing the Pdi 420oe densitometer supported by Quantity One software (Pdi, Huntington Station, NY).

Western Blot Analysis

The tissues were homogenized in lysis buffer containing 10 mM HEPES, pH 7.4, 350 mM sucrose, 5 mM EDTA, 0.2 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 mg/ml benzamidine, and 8 μg/ml calpain inhibitor I. Protein concentration was determined by Bradford assay (Bradford, 1976), using BSA as the standard protein. Protein fractions of 100 μg were subjected to immunoprecipitation for ETA receptor. ETA receptor was immunoprecipitated with rabbit specific polyclonal antibodies (final concentration of 2.5 μg/ml). Fractions of 30–80 μg protein were subjected to conventional Western blot analysis for ETB receptor. These protein samples were denatured by their boiling in 10% glycerol, 4.5% SDS, 5% β-mercaptoethanol, 62.5 mM Tris-base (pH 6.8), 1.5 mM EDTA, and 0.01% bromophenol blue for 5 min. The samples were then subjected to electrophoresis on a 12% agarose gel. Molecular weight markers were included in the gel. After electrophoresis, the separated proteins were electroblotted onto nitrocellulose membrane in buffer containing 20% methanol, 20 mM Tris-base (pH 8.3) and 150 mM glycine for 1.5 hr at room temperature. Transferred proteins were stained by Pounceau and destained in blocking buffer. Coomassie Brilliant Blue staining of SDS–PAGE gels was employed for evaluation of transfer efficiency.

Nitrocellulose membranes with transferred proteins were blocked by PBS (pH 7.5) solution, containing 10% non-fat milk and 0.1% Tween-20 for 2 hr at room temperature. PBS was renewed and nitrocellulose membranes were incubated with sheep specific polyclonal antibodies to ETA (final concentration of 12 μg/ml) and with rabbit specific polyclonal antibodies to ETB (final concentration of 1.5 μg/ml) for 14 hr at 4°C. Membranes were then washed extensively (four times, 15 min each) in PBS-Tween and incubated with HRP anti-rabbit IgG antibody or with HRP-donkey anti sheep IgG (final concentration of 16 ng/ml) for 1 hr. The membranes were then washed extensively (four times, 15 min each) in PBS-Tween and further subjected to ECL reagents. Immunoreactive proteins were visualized by 1–30 min exposure (according to the specific antibody) to a X-ray film and quantitated by densitometric analysis.

ET-1 Extraction and Determination

The tissues were extracted as described by Kitamura et al. (1989). Briefly, tissues were homogenized in 10 vol. of 1 M acetic acid, sonicated and boiled. Homogenates were centrifuged and the supernatants were loaded on C18 cartridges pre-equilibrated with 1 M acetic acid. The cartridges were washed with 1 M acetic acid and the adsorbed materials were eluted with 3 ml 60% acetonitrile in 0.1% trifluoroacetic acid. The eluates were evaporated under N_2 to dryness, dissolved in a minimum of 0.1 M acetic acid and then dissolved in ELISA work solution. ET-1 was determined by

commercial ELISA kit. The standard curve for ET-1 ranged from 2.9 to 94 fmol/ml. Cross-reactivities of ET-1 antiserum with ET-1, -2, -3, and big endothelin were 100, 100, <5, and <1%, respectively. The intra- and inter-assay coefficients of variation were 4 and 7%, respectively.

EIA for Progesterone

Serum P4 extraction was performed by using petroleum ether. Tissue culture medium P4 was assayed without extraction. ELISA plates were pre-coated with rabbit anti mouse immunoglobulins. Progesterone standards (1–100 ng/ml; 100 μl/well) or samples (100 μl/well) were diluted in assay buffer (0.05 M Tris, 0.5% BSA, 0.02 M NaCl, 0.05% Tween-20, pH 7.7) and incubated for 30 min at room temperature with 50 μl highly specific anti-progesterone monoclonal antibodies (final dilution 1:60,000). HRP-P4 (50 ng/100 μl/well) was then added for an additional 1 hr of incubation. TMB, dissolved in phosphate-citrate buffer, was used as a substrate in this assay. Each sample was analyzed in triplicates. The standard curve for P4 ranged from 3.12 to 100 ng/ml. The intra- and inter-assay coefficients of variation were 6.2 and 12%, respectively.

Statistical Analysis

Tissues for each experiment were derived from one individual rat. Experiments were repeated at least three times. For in vitro experiments, each point represents triplicates recovered from the indicated number of animals. Data are presented as means ± SEM. Statistical analysis was carried out by analysis of variance. Statistical significance of individual parameters was further examined by Student's *t*-test. Densitometric data was expressed as arbitrary density units, converted to percent from control, and compared as mentioned above. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Serum P4 at Pregnancy

In order to establish the pattern of luteal P4 secretion in our rat colony and to identify the stage of pregnancy at which rat CL aquires full function, serum P4 concentrations throughout pregnancy were determined. Serum P4 was initially elevated on the first day of pregnancy, reached maximal values on day 6 and remained high until day 22, just before delivery (data not shown).

The early CL of pregnancy in the rat is refractory to the luteolytic effect of $PGF_{2\alpha}$ (Wright et al., 1980). Administration of cloprostenol, a stable analog of $PGF_{2\alpha}$, on day 9 of pregnancy significantly decreased P4 concentration in the serum within 40 min ($P < 0.04$). A 50% inhibition ($P < 0.0001$) was demonstrated at 1 hr after injection (Fig. 1).

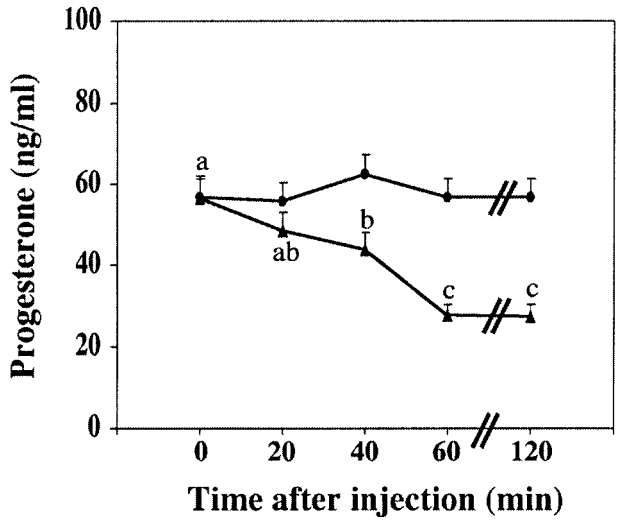

Fig. 1. Serum progesterone concentrations after administration of cloprostenol. Cloprostenol (0.025 mg/rat) was administrated on day 9 of pregnancy and progesterone concentrations were determined at the indicated time points as described in Materials and Methods. Data are presented as mean ± SE of the results of five experiments. Between time points, values with different letters represent significant difference.

ET-1 Expression in CL of Pregnant Rats

To elucidate the physiological relevance of the involvement of ET-1 in $PGF_{2\alpha}$ action, the effect of cloprostenol on the expression of mRNA for ET-1 and its peptide product in rat CL of day 9 of pregnancy was studied by RT-PCR and ELISA, respectively. We found that the expression of mRNA for ET-1 in corpora lutea of pregnant rats was elevated by 70% at 1 hr ($P < 0.04$) and continued to increase by 2 hr (113%, $P < 0.02$, Fig. 2) after cloprostenol administration.

In accordance with mRNA levels, the ET-1 peptide concentration in rat CL on day 9 of pregnancy was elevated from 2.91 ± 0.07 fmol/mg before treatment to 11.82 ± 5.8 fmol/mg ($P < 0.05$) at 2 hr after cloprostenol administration (Fig. 3). A similar elevation of ET-1 concentrations were observed at parturition, the time of spontaneous luteolysis (8.13 ± 2.0 fmol/mg; $P < 0.02$).

ET Receptors Gene Expression in CL of Pregnant Rats

The effect of $PGF_{2\alpha}$ on the expression of the two types of endothelin receptors, ETA and ETB in rat CL was also studied. We found that CL of rat on day 9 of pregnancy expresses mRNA for both ETA and ETB receptors. The amount of the mRNA of these receptors was not affected at the first 2 hr after cloprostenol administration (Fig. 4A,B).

Western blot analysis was utilized in order to evaluate the expression of ETA and ETB receptor proteins. These experiments detected a 47-kDa ETA and an ~40-kDa ETB receptor types in rat corpora lutea (Fig. 5). Incubation of each of these antibodies with an excess of their corresponding recombinant peptide eliminated their immunoreactivity with the above mentioned proteins. Cloprostenol did not seem to affect the expression of the ET receptors at the first 2 hr after administration (Fig. 5).

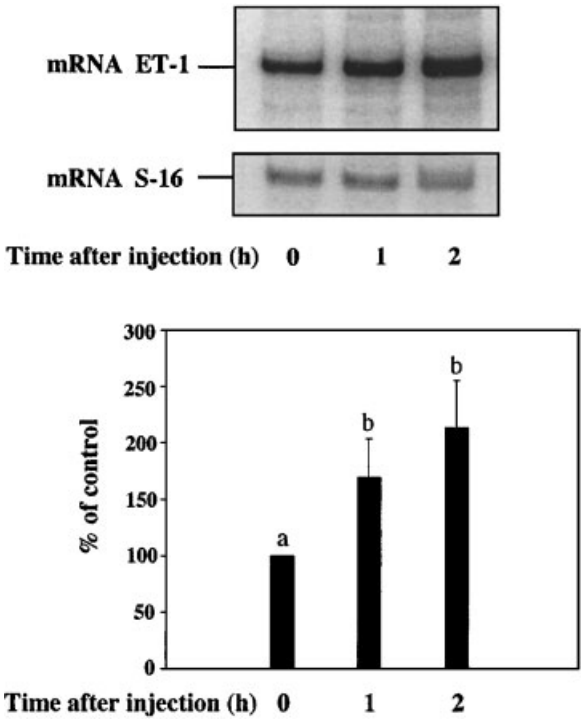

Fig. 2. ET-1 expression in CL of pregnant rats. Corpora lutea were collected at 0, 1, and 2 hr after a single injection of cloprostenol (0.025 mg/rat). For each experiment, RNA was isolated from a pool of corpora lutea recovered from one individual rat on day 9 of pregnancy at the indicated times after injection of cloprostenol and subjected for RT-PCR analysis as described in Materials and Methods. **Upper panel**: The results of RT-PCR analysis from one representative experiment are presented. **Lower panel**: Quantitation of the results obtained from four individual such experiments (a total of 12 rats were examined). Data are presented as mean ± SE. Between time points, values with different letters represent significant difference.

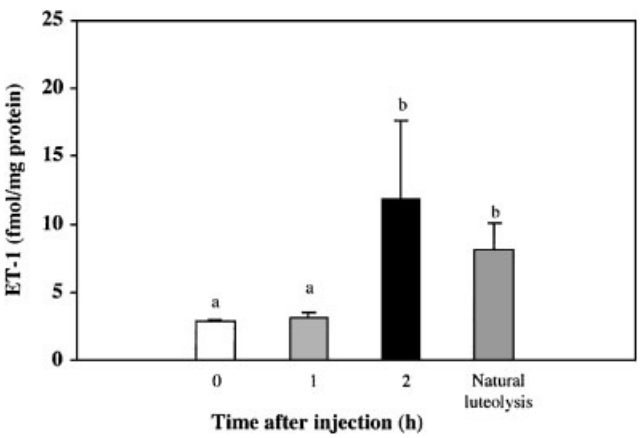

Fig. 3. ET-1 content in CL of pregnant rats administrated with cloprostenol or at parturition. Corpora lutea were collected from rats on day 9 of pregnancy at 0, 1, and 2 hr after a single injection of cloprostenol (0.025 mg/rat) or on day 22, before parturition. For each experiment, peptide fraction was extracted from a pooled corpora lutea recovered from one individual rat and subjected to ELISA as described in Materials and Methods. Data are presented as mean ± SE of three individual experiments (a total of twelve rats were examined). Between time points, values with different letters represent significant difference.

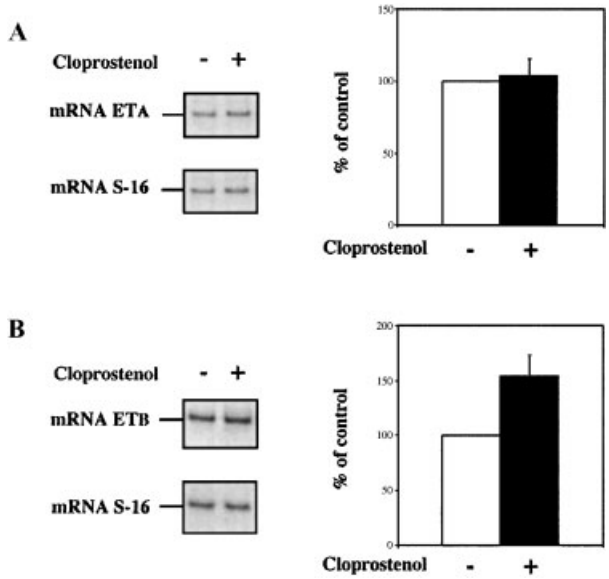

Fig. 4. ET receptors gene expression in corpora lutea of pregnant rats. (**A**) ETA receptor type; (**B**) ETB receptor type. Corpora lutea were collected from rats on day 9 of pregnancy before (cloprostenol −) and at 1 hr (cloprostenol +) after a single injection of cloprostenol (0.025 mg/rat). For each experiment, RNA was isolated from a pool of corpora lutea recovered from one individual rat and subjected to RT-PCR analysis as described in Materials and Methods. Data are presented as mean ± SE of five individual experiments.

Effect of $PGF_{2\alpha}$ and ETA Type Receptor-Specific Antagonist on P4 Secretion In Vitro

Demonstration of ET-1 and its receptors in rat CL and the effect of $PGF_{2\alpha}$ on ET-1 expression by this tissue suggest that the ET system may be involved in the $PGF_{2\alpha}$-induced luteolysis in the rat. If indeed $PGF_{2\alpha}$

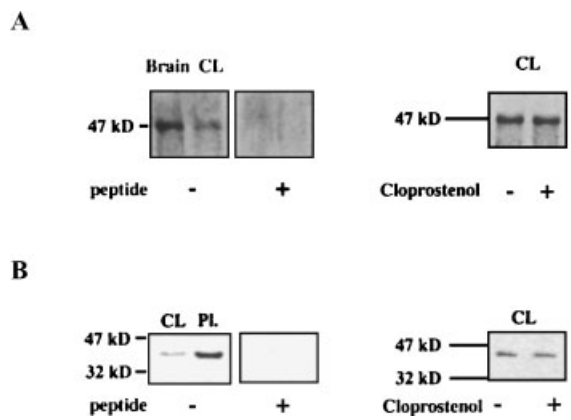

Fig. 5. ET receptors protein expression in corpora lutea of pregnant rats. (**A**) ETA receptor type. (**B**) ETB receptor type. Corpora lutea were collected from rats on day 9 of pregnancy before (cloprostenol −) and at 1 hr (cloprostenol +) after a single injection of cloprostenol (0.025 mg/rat). For each experiment, protein was extracted from a pool of corpora lutea recovered from one individual rat and subjected to Western blot analysis as described in Materials and Methods. Specificity of antibodies was confirmed by preincubation with a recombinant peptide for each receptor type (peptide +). Pl, placenta. Data are presented as mean ± SE of five individual experiments.

action is mediated by ET-1, then interference with ET-1 ligand binding to its receptor should inhibit the effect of $PGF_{2\alpha}$. This assumption was tested in isolated intact rat corpora lutea incubated in vitro with LH, $PGF_{2\alpha}$, and their combinations in the presence or absence of BQ123, a selective antagonist to the ETA receptor. Progesterone determined in the culture medium was used as a parameter for CL function.

LH (100 ng/ml) stimulated P4 secretion approximately by threefold ($P < 0.001$; Fig. 6). The effect of LH in this system was totally abolished by the addition of $PGF_{2\alpha}$ ($P < 0.002$). Under these conditions, the ETA receptor antagonist BQ123 (10^{-7} M) prevented the antisteroidogenic effect of $PGF_{2\alpha}$ ($P < 0.001$).

VEGF Gene Expression in CL of Pregnant Rats

In addition to the ET-1-mediated $PGF_{2\alpha}$ antisteroidogenic effect, $PGF_{2\alpha}$ may activate ET-1 and its receptor to induce vasoconstriction. Vasospasm of the CL can result in hypoxia of this tissue that may contribute to termination of its function. To test this possibility, we analyzed corpora lutea of rats administrated with cloprostenol on day 9 of pregnancy for the expression of VEGF, as a marker of hypoxia. RT-PCR analysis indeed revealed an elevated expression of mRNA for VEGF in the CL of pregnancy (Fig. 7) as soon as 1 hr after cloprostenol administration ($P < 0.02$). No further increase was observed at 2 hr ($P < 0.01$).

DISCUSSION

Our study suggests that ET-1 is involved in the disruption of luteal function by $PGF_{2\alpha}$ in a pregnant rat. It further raises the possibility that the ET-1-mediated $PGF_{2\alpha}$-induced luteolysis may involve vasoconstriction, followed by development of hypoxia in the luteal tissue. These conclusions are based on our experimental evidence that $PGF_{2\alpha}$-induced luteolysis is accompanied by increased expression of luteal ET-1 mRNA and protein. It is further supported by the demonstrated reversal of $PGF_{2\alpha}$ inhibition of progesterone output by an ETA receptor antagonist. It also takes into account the $PGF_{2\alpha}$-induced luteal expression of VEGF mRNA.

Previous studies that tested the $PGF_{2\alpha}$ luteolytic effect in the rat, used the immature superovulated and/or the pseudopregnant animal models. The superovulated rat model represents the CL of the estrous cycle. In contrast to the pregnant rat in which luteolysis is induced by $PGF_{2\alpha}$, in the cycling rat the CL regresses gradually due to the lack of luteotrophic support (Long and Evans, 1992). The fact that $PGF_{2\alpha}$ is dispensable in cycling rodents was manifested in the $PGF_{2\alpha}$ receptor knockout animal model (Sugimoto et al., 1997). This $PGF_{2\alpha}$ receptor-deficient mice did not show

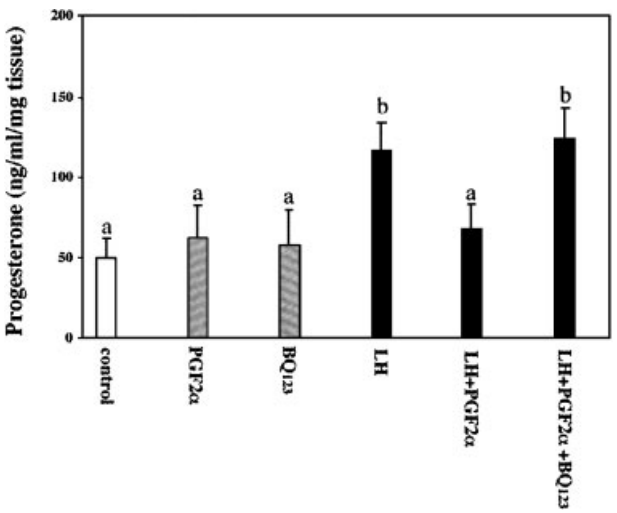

Fig. 6. Effect of ETA receptor specific antagonist on $PGF_{2\alpha}$-induced inhibition of progesterone secretion in vitro. Corpora lutea were recovered on day 9 of pregnancy, incubated overnight with control media or with $PGF_{2\alpha}$ (1 μg/ml), the specific ETA receptor antagonist BQ123 (10^{-7} M), oLH (100 ng/ml), and their combinations. At the end of the incubation period media were collected and progesterone concentrations were determined by ELISA as described in Materials and Methods. Data are presented as mean ± SE of seven individual experiments. Values with different letters represent significant difference.

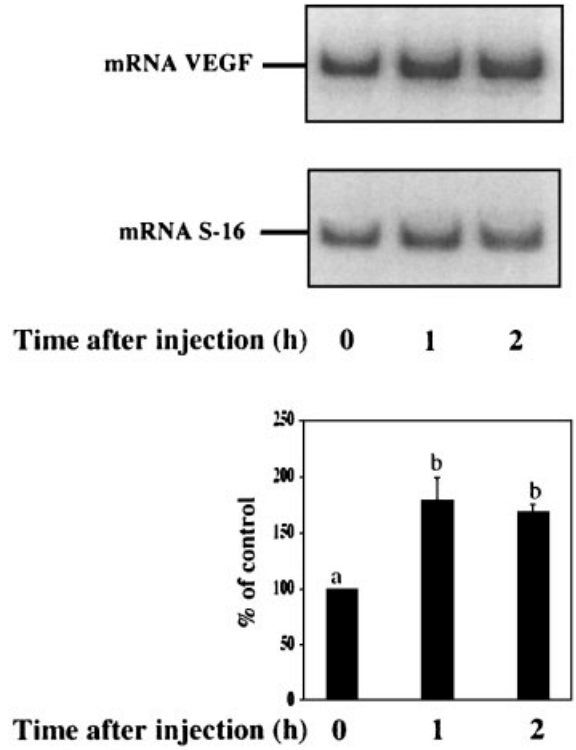

Fig. 7. VEGF expression in CL of pregnant rats administrated with cloprostenol. Corpora lutea were collected at 0, 1, and 2 hr after a single injection of cloprostenol (0.025 mg/rat). For each experiment, RNA was isolated from a pool of corpora lutea recovered from one individual rat on day 9 of pregnancy at the indicated times after injection of cloprostenol and subjected for RT-PCR analysis as described in Materials and Methods. **Upper panel**: The results of RT-PCR analysis from one representative experiment are presented. **Lower panel**: Quantitation of the results obtained from four individual experiments. Data are presented as mean ± SE of four experiments. A total of 12 rats were examined. Between time points, values with different letters represent significant difference from control.

abnormality in the estrous cycle, ovulation, fertilization, or implantation, but failed to exhibit the decline in serum P4 concentration that preceeds parturition. In the pseudopregnant rat model cervical stimulation provokes prolactin secretion that exerts in turn its luteotrophic effect to form a fully functional CL. However, in this last animal model, the lifespan of the CL is relatively short and placenta does not develop. Since placental lactogen participates in regulation of rat CL, the absence of the placenta represent an obvious disadvantage. Therefore, the present study utilized the physiological model of fully developed CL of pregnancy to analyze the luteolytic effect of $PGF_{2\alpha}$ in this animal species. Furthermore, our experiments were conducted at two complementary levels. Analysis of the in vivo response of the $PGF_{2\alpha}$-treated pregnant rat was complemented by studies performed in vitro, in corpora lutea isolated from rats at the exact same stage of pregnancy. This culture system of intact corpora lutea represents very closely the physiological conditions, both in terms of tissue composition as well as its architecture. Specifically, the spherical architecture of the CL is not disturbed. Moreover, the proportion between the small, large, and endothelial luteal cells is maintained. Furthermore, anatomical interactions between these cellular components established by gap junctions and extracellular matrix is fully preserved.

Using this model, we initially confirmed the luteolytic effect of $PGF_{2\alpha}$ both in vivo, in the intact pregnant animal, and in vitro, in the isolated corpora lutea. The rapid decline in P4 production demonstrated in both our experimental systems is consistent with previous findings generated in immature superovulated and/or pseudopregnant rats (Riley and Behrman, 1991; Sawada and Carlson, 1991; Fiedler et al., 1999), as well as in the cow (Juengel et al., 1993), ewe (McCracken et al., 1972), monkey (Summers et al., 1985), and human (Wentz and Jones, 1973). This decline in P4 secretion represents early stages of luteal regression (Juengel et al., 1993) that is physiologically initiated by $PGF_{2\alpha}$ surges (McCracken et al., 1972; Wentz and Jones, 1973; Baird et al., 1976). The response to $PGF_{2\alpha}$ is elicited through the high-affinity receptors for this hormone demonstrated in rats, as well as in ovine and bovine corpora lutea (Powell et al., 1974; Rao, 1975; Orlicky et al., 1992). The affinity and capacity of these $PGF_{2\alpha}$ receptors in the rat do not change between day 4 and 10 of pseudopregnancy (Wright et al., 1980) and their activation, as shown herein, induces luteolysis.

Endothelial cells from different tissues, including the luteal microvascular system (Girsh et al., 1996b), produce ET-1 (Yanagisawa et al., 1988; Hexum et al., 1990; Prasad et al., 1991) that acts apparently through binding to its relevant receptor. Several ETs receptor types, characterized by different pharmacological and binding properties of the various ET isopeptides, are present in mammalian tissues (Williams et al., 1991). It was shown that ETB receptor mRNA is expressed by endothelial cells of the human CL (Karam et al., 1999). High affinity ETA receptors were found on steroidogenic cells from bovine luteal tissue and P4 secretion from these cells was inhibited by ET-1 in a dose-dependent manner (Girsh et al., 1996a). Similar findings were reported later for human purified luteal cells (Apa et al., 1998). In the present study, we demonstrate that rat corpora lutea express mRNA for both ETA and ETB receptors and their corresponding proteins. Several studies suggested that the ETA receptors mediate vasoconstriction and that the ETB receptors mediate vasodilation (Vane, 1990).

It was reported in the cow that $PGF_{2\alpha}$-induced luteolytic effect involves the induction of ET-1 expression in luteal endothelial cells (Girsh et al., 1996b). In the present study, we extend these findings to include the pregnant rat. Our interest in the rat model is provoked by the unique characteristics of the CL of this animal species. Unlike the other mammalian species that spontaneously develop a luteal phase after ovulation, rat postovulatory corpora lutea require a luteotropic support in order to become functional. Confirming previous findings in the cow, our findings suggest that the involvement of ET-1 in $PGF_{2\alpha}$-induced luteolysis represents a universal mechanism that is apparently shared by rodents as well.

The CL is characterized by an extensively developed capillary network required to sustain its high metabolic requirement. Since ET-1 is a potent vasoconstrictor, we assumed that in addition to its antisteroidogenic effect, the ET-1 mediated $PGF_{2\alpha}$ action may, in turn, create hypoxic conditions in the CL that possibly participate in luteolysis. Indeed, it has been previously reported that in the ewe $PGF_{2\alpha}$ induces luteal vasoconstriction (Ford et al., 1979). On the other hand, a later study demonstrated an increase in blood flow in bovine CL undergoing luteolysis (Miyamoto et al., 1997b). However, a most recent publication from this same laboratory (Acosta et al., 2002) shows that the initial increase in blood flow observed after $PGF_{2\alpha}$ administration is followed by a continuos decrease clearly associated with the progression of luteolysis. This last observation seems to agree with our assumption that $PGF_{2\alpha}$ may elicit hypoxia in the luteal tissue. Supporting this idea, we herein demonstrate that the expression of VEGF mRNA, which is one of the primary characteristics of hypoxic conditions (Popovici et al., 1999; Pilch et al., 2001) is significantly elevated after cloprostenol treatment.

Collectively, results reported in this study indicate the physiological relevance of ET-1 in rat CL function and postulate its involvement in $PGF_{2\alpha}$-induced luteolysis in this animal species. These findings further suggest that, in addition to its antisteroidogenic effect, ET-1 mediated $PGF_{2\alpha}$-induced luteolysis may possibly stimulate luteal vasoconstriction that participates in termination of CL function.

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