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Prostaglandin $F_{2\alpha}$ Regulation of the Bovine Corpus Luteum Endothelin System During the Early and Midluteal Phase[1]

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ABSTRACT

Recent evidence in the cow suggests that endothelin-1 (ET-1) plays a role during prostaglandin (PG) $F_{2\alpha}$-induced luteal regression. We have examined the effects of treatment with $PGF_{2\alpha}$ during the early and midluteal phases on three components of the endothelin system: endothelin-converting enzyme-1 (ECE-1), ET type A receptor (ET_A), and ET-1 in the bovine corpus luteum (CL). Cyclic beef cows were injected (0 h) on Day 4 or 10 with either saline or the $PGF_{2\alpha}$ analogue Lutalyse (15 mg). The CL were collected at 2 (n = 11), 10 (n = 23), 24 (n = 15), or 48 h (n = 12) after treatment. The cows in which CL were removed after 10 h comprised of two experimental groups. The first group (n = 11) received one injection; the second group (n = 12) received two injections, one at 0 h and one at 8 h. The cows in which CL were collected after 24 and 48 h received one injection every 8 h. Semiquantitative reverse transcriptase-polymerase chain reaction was used to evaluate the mRNA encoding ECE-1, ET_A, and ET-1. The ECE-1 and ET_A proteins were evaluated by semiquantitative Western blot analysis. The ET-1 was the most likely component of the endothelin system target for $PGF_{2\alpha}$ regulation during the midluteal phase. The ET_A and ECE-1 genes were constitutively expressed in the Day 4 and Day 10 CL. A practical application of this observation is that it may be possible to target the ET-1 gene as a way to manipulate the luteolytic action of $PGF_{2\alpha}$.

corpus luteum, corpus luteum function, gene regulation, ovary, ovulatory cycle, progesterone

INTRODUCTION

Progesterone (P_4), secreted by the corpus luteum (CL), is essential for the maintenance of pregnancy in the cow. When normal pregnancy occurs, the CL continues to secrete P_4 throughout pregnancy. If pregnancy does not occur, the CL undergoes luteolysis, P_4 secretion declines, and consequently, the next ovarian cycle occurs [1, 2].

Luteolysis is brought about by the direct actions of prostaglandin (PG) $F_{2\alpha}$ on the CL. A combination of $PGF_{2\alpha}$-induced changes result in cessation of luteal P_4 production and involution of luteal tissues with luteal cell death [3–5]. The luteolytic actions of $PGF_{2\alpha}$ are mediated by specific plasma membrane receptors that belong to the seven transmembrane G protein-coupled superfamily [6–10]. Luteal regression might involve endocrine as well as paracrine/autocrine actions of $PGF_{2\alpha}$ [3, 5].

Interestingly, for those species in which $PGF_{2\alpha}$ is luteolytic, the early CL is resistant to the luteolytic actions of $PGF_{2\alpha}$. Insensitivity to $PGF_{2\alpha}$ has been observed in cows [11–14], marmoset monkeys [15], pigs [16], and rats [17]. The mechanisms responsible for this insensitivity of early CL to the luteolytic actions of $PGF_{2\alpha}$ are poorly understood; however, this insensitivity is not due to the lack of high-affinity receptors for $PGF_{2\alpha}$ [6, 18, 19]. Indeed, recently, it has become clear that $PGF_{2\alpha}$ can regulate cellular and molecular processes, such as ascorbic acid depletion and inhibition of mRNA for steroidogenic enzymes and PG receptors in CL at the early and midluteal phase [20].

Clearly, luteolysis is a complex process involving changes in the expression of many genes in at least three cell populations of the CL: the large and small steroidogenic cells, and the endothelial cells [21–24]. Reports have appeared that either natural luteolysis or treatment with $PGF_{2\alpha}$ may decrease or increase mRNA encoding several pertinent genes [20].

Recent evidence in the cow suggests that endothelin-1 (ET-1) plays an essential role during $PGF_{2\alpha}$-induced luteal regression [23]. Both ET-1 mRNA and peptide were elevated within 2 h after in vivo or in vitro treatment with $PGF_{2\alpha}$ [25, 26]. High concentrations of ET-1 inhibit P_4 production by luteal cells [23]. The inhibitory effects of ET-1 have been shown to be exerted via the selective ET type A receptors (ET_A) [25]. Indeed, administration of $PGF_{2\alpha}$ during the midluteal phase induced both ET-1 and ET_A [21]. In contrast, during the early luteal phase, administration of $PGF_{2\alpha}$ did not alter the expression of ET-1 and ET_A [21]. These observations led Levy et al. [21] to suggest that the lack of ET-1 synthesis and response during the early luteal phase may render early CL insensitive to $PGF_{2\alpha}$.

A 21-amino acid peptide, ET-1 is a member of the endothelin family that includes ET-2 and ET-3 [27]. Endothelins are initially synthesized as 203-amino acid precursor proteins, called preproETs (ppET), that are first proteolytically cleaved to generate big ETs and are then processed to the active peptides via an endothelin-converting enzyme (ECE) [28, 29]. ECE-1 is the key enzyme in cleaving the inactive, big ET-1 at the Trp^{21}/Val^{22} bond for its conversion to the active ET-1 peptide [30, 31]. In fact, ECE-1 has been shown to be present in both endothelial and steroidogenic cells of the bovine CL [32].

The ETs act on at least two distinct ET receptor subtypes of the seven transmembrane G protein-coupled receptors: ET_A, and ET type B receptor (ET_B). Both ET-1 and ET-2 bind to ET_A with higher affinity than ET-3 [33]. However, ET_B binds all three isopeptides with equal affinity [34]. In

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the cow, ET_A has been identified in CL at the midluteal phase [25]. The ET_A mRNA expression was demonstrated on small and large luteal cells and on endothelial cells of the bovine CL [23].

In the present study, we have examined the effects of administration of $PGF_{2\alpha}$ during the early and midluteal phases on the mRNA expression encoding ECE-1, ET_A, and ppET-1 and on the amounts of ET_A and ECE-1 protein in bovine CL.

MATERIALS AND METHODS

Treatments and Tissue Collection

Cyclic beef cows were treated on Day 4 (n = 31) or Day 10 (n = 30) with either saline or $PGF_{2\alpha}$ (15 mg; Lutalyse; Pharmacia and Upjohn, Inc., Kalamazoo, MI). Ovaries were collected by blunt dissection after ovariectomy via supravaginal incision under epidural anesthesia (6–9 ml of 2% lidocaine for cows weighing 450–700 kg) [35, 36]. The ovaries were removed at 2 (n = 11), 10 (n = 23), 24 (n = 15), or 48 h (n = 12). The experimental group in which the ovaries were removed at 2 h received a single injection. The cows in which the ovaries were removed at 10 h included two experimental groups: one that received a single injection at time zero (0 h, n = 11), and one that received two injections (one at 0 h and one at 8 h). The experimental groups in which the ovaries were collected at 24 and 48 h received an injection every 8 h. The CL were immediately dissected from the collected ovaries, frozen in liquid nitrogen, and stored at −70°C. Protocols for all animal experiments described in this study were approved by the West Virginia University Animal Care and Use Committee.

Total RNA Isolation and Primer Design

Total RNA was isolated from tissue samples with Trizol reagent according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). The RNA was quantified spectroscopically at 260 nm. The primers for ECE-1 (Table 1) were designed based on known DNA sequences of human and bovine ECE-1 and generated a 500-base pair (bp) polymerase chain reaction (PCR) product. The 500-bp ECE-1 cDNA was sequenced (Davis Sequencing, Davis, CA) and verified to represent genuine ECE-1α isoform. The sequence was used to search the nucleic acid database at the National Center for Biotechnology Information (NCBI) with the BLAST software [37]. The ET_A primers (Table 1) were designed based on known ET_A DNA sequences. The PCR product generated for ET_A was 500 bp. It was sequenced (Davis Sequencing) and verified to represent genuine ET_A receptor via NCBI BLAST [37]. The primers for ET-1 were those designed by Levy et al. [21]. These primers (Table 1) have been verified to amplify genuine ET-1 [21]. We have used their sequence to search the nucleic acid database at NCBI with the BLAST software [37] and corroborated their specificity. Primers for two constitutively expressed genes, β-actin (Promega, Madison, WI) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were used as internal standards (Table 1).

Semiquantitative Reverse Transcriptase-PCR

Semiquantitative reverse transcriptase (RT)-PCR assays were performed using the OneStep RT-PCR Kit (Qiagen, Valencia, CA). The parameters for the RT-PCR assays were optimized for semiquantitative analysis of expression of ECE-1, ET_A, ET-1, β-actin, and GAPDH transcripts in the bovine CL.

The number of cycles for ECE-1 was tested in the range of 20–30 cycles with a total RNA concentration of 600 ng/μl (data not shown). Total RNA concentration for ECE-1 and β-actin was tested in the range of 5–50 ng/μl (data not shown). When optimal parameters were determined for ECE-1 and β-actin, the RT-PCR assays were performed at 50°C for 30 min for reverse transcription reaction and at 95°C for 15 min for activation of RT-PCR enzyme. Thirty cycles were carried out as follows: 94°C for 30 sec for denaturing, 52°C for 1 min for annealing, and 72°C for 1 min for extension, followed by a 10-min final extension at 72°C. The β-actin housekeeping gene was determined to be beyond the linear range for the optimal total RNA concentration for ET_A (data not shown). Therefore, GAPDH was used as the internal standard for ET_A.

The number of cycles for ET_A was tested in the range of 20–28 cycles at 600 ng/μl (data not shown). Total RNA concentration for ET_A and GAPDH was tested in the range of 30–600 ng/μl (data not shown). When optimal parameters were determined, the RT-PCR assays were performed using 50°C for 30 min for reverse transcription reaction and 95°C for 15 min for activation of RT-PCR enzyme. Twenty-five cycles for GAPDH and 28 cycles for ET_A were carried out as follows: 94°C for 50 sec for denaturing, 52°C for 1 min for annealing, and 72°C for 1 min for extension, followed by a 10-min final extension at 72°C.

TABLE 1. Primers list.

Gene		Primers
β-Actin	Sense:	5′-TCATGAAGTGTGACGTTGACATCCGT-3′
	Antisense:	5′-CCTAGAAGCATTTGCGGTGCACGATG-3′
ECE-1	Sense:	5′-GATGGTGATGTTGGCCAGCG-3′
	Antisense:	5′-GGGAACACAACCAGGCCATC-3′
GAPDH	Sense:	5′-TGTTCCAGTATGATTCCACCC-3′
	Antisense:	5′-TCCACCACCCTGTTGCTGTA-3′
ET_A	Sense:	5′-TTCTACTTCTGCATGCCCCTGGT-3′
	Antisense:	5′-GTCCTTGTGGCTGCTCCTCTCAG-3′
ET-1	Sense:	5′-TGTCTTCATCAGCAGCTCG-3′
	Antisense:	5′-GTTTCTCCCTGAAATGTGCC-3′

The number of cycles for ET-1 was tested in the range of 20–30 cycles at 100 ng/μl (data not shown). When optimal parameters were determined, the RT-PCR assays were performed using 50°C for 30 min for reverse transcription reaction and 95°C for 15 min for activation of RT-PCR enzyme. Twenty-six cycles for GAPDH and 30 cycles for ET-1 were carried out as follows: 94°C for 50 sec for denaturing, 52°C for 1 min for annealing, and 72°C for 1 min for extension, followed by a 10-min final extension at 72°C.

All PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and viewed using the Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA). Data were collected using the densitometric analysis of the Quantity One quantitation software package (version 4.0; Bio-Rad Laboratories).

Western Blotting Analyses of ECE-1 and ET_A

Protein samples from saline- and $PGF_{2\alpha}$-treated, Day 4 and Day 10 bovine CL collected after 24 and 48 h were obtained from the tissues collected as described in *Treatments and Tissue Collection.* Proteins were isolated from individual bovine CL using the Trizol reagent as specified by the manufacturer. Protein concentration in the samples was determined with the Bio-Rad Protein Assay. The optimal protein concentration needed to detect ECE-1, ET_A, and actin was determined by testing 5, 10, 20, 50, and 100 μg of isolated proteins from the CL.

The SDS-PAGE was carried out as previously described [38]. Briefly, protein samples were loaded onto an 8% polyacrylamide gel. After electrophoresis at 150 V for 1 h, the resolved proteins were transferred to polyvinylidene fluoride membranes (Biotechnology Systems, Boston, MA) using a Mini-V 8.10 Blot Module (Gibco BRL). The membranes were blocked in 1% BSA with 0.05% Tween-20 in Tris-buffered saline (TBS-T, pH 7.5) for 2 h at room temperature. The membranes were incubated with the primary antibody for 2 h at room temperature and washed three times for 10 min in TBS-T. Subsequently, the membranes were incubated with anti-rabbit (1:5000 [v/v]; Amersham Pharmacia Biotech, Piscataway, NJ) or anti-mouse (1:30 000 [v/v]; Gibco, Grand Island, NY) horse radish peroxidase-conjugated antibodies for 1 h. After three TBS-T washes, visualization was achieved using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and Kodak Biomax Light Film (Eastman Kodak Company, Rochester, NY). This technology was selected because of its versatility in allowing the stripping of primary and secondary antibodies from membranes for sequential reprobing of membranes with a variety of antibodies.

Stripping of membranes was performed with Restore Western Blot Stripping Buffer (Pierce, Rockport, IL). Stripping conditions were tested to demonstrate complete removal of antibodies, and 1 h at room temperature was found to be effective for all antibodies used in this study. Sequential reprobing with the same antibody demonstrated that the signal intensity did not decrease during up to five strippings of the membranes (data not shown). Images of the detected proteins were captured using the Fluor-S MultiImager. Densitometry of the bands of interest were performed using the Quantity One quantitation software. The intensity of the signal corresponding to the ECE-1 and ET_A proteins were standardized by the corresponding intensity of the actin protein control. This normalization of data allowed us to estimate, in a semiquantitative manner, the amount of protein in the sample of interest. This semiquantitative Western blot methodology has been described earlier [39]. Three primary antibodies

were used in these studies. A mouse anti-actin monoclonal antibody (Chemichon International, Inc., Temecula, CA) was used at a dilution of 1:3000 (v/v). The rabbit anti-ET_A polyclonal antibody (Chemichon International, Inc.) was used at a dilution of 1:200 (v/v). The rabbit polyclonal anti-bovine ECE-1 antibody (a generous gift of Dr. Masashi Yanagisawa from the Howard Hughes Medical Institute, University of Texas Southwestern Medical Institute, Dallas, TX) was used at a dilution of 1:2000 (v/v). The specificity of each primary antibody was confirmed using antibody-specific epitope peptides preincubated with the primary antibody before addition to membranes.

Statistical Analysis

Statistical analyses were performed using JMP, a statistical software program for the Apple Macintosh [40]. For the RT-PCR, semiquantification was based on the ratios of the densitometric analyses of ECE-1 to β-actin and of ET_A to GAPDH. Thirty-one samples were used for Day 4, and 30 samples were used for Day 10. The average values for each time point were calculated, and saline control versus $PGF_{2\alpha}$ treatments were compared. For the Western blot analysis, semiquantification was based on the ratios of the densitometric analyses of ECE-1 and ET_A to actin. Fifteen samples were used for Day 4, and 12 samples were used for Day 10. The average values for each time point were calculated, and saline controls versus $PGF_{2\alpha}$ were compared. Data are presented as the mean ± SEM. Statistical significance was determined using the Student *t*-test [40].

RESULTS

Detection of ECE-1, β-Actin, ET_A, ET-1, and GAPDH Transcripts in Bovine CL

The validity of the RT-PCR assays for semiquantitative evaluation was supported by at least three criteria: 1) by selecting, for each target, an RNA concentration yielding amplification in the exponential phase of the RT-PCR (data not shown); 2) by repetitive observations of results within experimental groups (at least two assays per data point); and 3) by using an appropriate standard control.

The RT-PCR assays were optimized for semiquantitative analysis of expression of ECE-1, β-actin, ET_A, ET-1, and GAPDH transcripts in bovine CL. In the case of ECE-1, plotting the intensity of the RT-PCR signal (as expressed by absolute optical density values) against the number of amplification cycles revealed a linear relationship between cycles 24 and 30 ($r^2 = 0.61$; data not shown). The PCR products generated for β-actin and ECE-1 were 285 and 500 bp, respectively, with primer pairs 1 and 2 (Table 1). The 500-bp band was sequenced and shown to be genuine ECE-1 (data not shown). The intensity of the band produced with the primer pair corresponding to β-actin was stronger than the one produced with the primer pair corresponding to ECE-1. However, the intensity of the bands produced was well correlated with the amount of total RNA tested (range, 5–50 ng). Based on this finding, in subsequent RT-PCR experiments, the total RNA concentration used to evaluate the expression of these two genes was selected at 10 ng. The number of cycles for the RT-PCR was fixed at 30 cycles, and under these conditions, the level of expression of both genes was in the linear range.

The amount of gene expression for β-actin was much greater than that for the ET_A (data not shown). The GAPDH gene was a better internal standard than β-actin when evaluating the amount of mRNA encoding the ET_A. As expected, the primer pairs designed for GAPDH and ET_A (primer pairs 3 and 4) (Table 1) generated RT-PCR products of 900 and 500 bp, respectively. These RT-PCR products were sequenced and shown to represent genuine ET_A and GAPDH (data not shown). The optimal number of cycles for ET_A was fixed at 28, because a good correlation was found between the signal generated when the cycles were tested from 22 to 28 cycles ($r^2 = 0.60$). Because the GAPDH gene was expressed at a higher number of copies than the ET_A gene (data not shown), the number of cycles for GAPDH was selected at 25. When optimizing the RT-PCR conditions for ET_A and GAPDH, good correlation was found (ET_A, $r^2 = 0.64$; GAPDH, $r^2 = 0.61$) between the intensity of the bands produced and the amount of total RNA used (range, 30–600 ng). The primer pair for ET-1 generated a RT-PCR product of 580 bp. The optimal number of cycles determined was 30 for ET-1 ($r^2 = 0.98$) and 26 for GAPDH ($r^2 = 0.96$, data not shown). Total RNA concentration was fixed at 100 ng.

Detection of ECE-1, Actin, and ET_A Proteins in Bovine CL

Single bands of approximately 130, 57, and 43 kDa were detected when the protein samples were loaded at concentrations of 10 μg or greater; at lower protein concentrations, no bands were detected (data not shown). The molecular weights of the detected proteins corresponded closely to the published size bands detected for ECE-1, ET_A, and actin [41, 42] by their respective antibodies. Based on this finding, we chose 30-μg protein samples to evaluate the effect of $PGF_{2\alpha}$ on the amount of these proteins in the samples. The specificity of ET_A antibody was confirmed by preabsorption of the antibody with excess peptide (data not shown). The specificity of the bands detected with the ECE-1 and actin antibodies have been similarly confirmed elsewhere [41, 42].

Effect of $PGF_{2\alpha}$ on Luteal ECE-1 mRNA

The amounts of luteal mRNA encoding ECE-1, ET_A, and ET-1 were evaluated in the early and midluteal phase of the cycle at 2 and 10 h after a dosage of 15 mg of $PGF_{2\alpha}$. The sensitivity of the early and midluteal CL was evaluated further by the response to repeated injections of 15 mg of $PGF_{2\alpha}$ every 8 h.

As reported elsewhere [43], the concentration of $PGF_{2\alpha}$ used in this study was effective in reducing plasma concentration of P_4 in Day 4 and Day 10 CL. Figure 1A shows a representative photograph of the RT-PCR products obtained with total RNA from the Day 4 CL collected after 48 h from the group receiving multiple injections. Lanes 1–4 represent samples from cows treated with saline. Lanes 5–8 represent samples from cows treated with $PGF_{2\alpha}$. Figure 1B shows a representative photograph of the RT-PCR products obtained with total RNA from the Day 10 samples. The tissues were collected after 48 h. Lanes 1–6 represent samples from cows treated with saline. Lanes 7–10 represent animals treated with $PGF_{2\alpha}$. Figure 1C shows that, in the early CL, $PGF_{2\alpha}$ treatment had no effect on the amount of mRNA encoding ECE-1 at any time point examined ($P > 0.1$). After 48 h, a slight decrease was observed in the amount of ECE-1 mRNA, but this difference was not statistically significant from its saline control. This finding was also seen at the protein level.

During the midluteal phase of the cycle, one or multiple injections of $PGF_{2\alpha}$ had no effect on the amount of mRNA encoding ECE-1 up to 10 h ($P < 0.05$) (Fig. 1C). In contrast, after 24 h, $PGF_{2\alpha}$ had induced a significant ($P = 0.05$), 20% reduction in the amount of ECE-1 mRNA. By 48 h, this $PGF_{2\alpha}$-induced inhibition was more pronounced (~60%, $P < 0.01$). This effect of $PGF_{2\alpha}$ was also observed in the amount of ECE-1 protein.

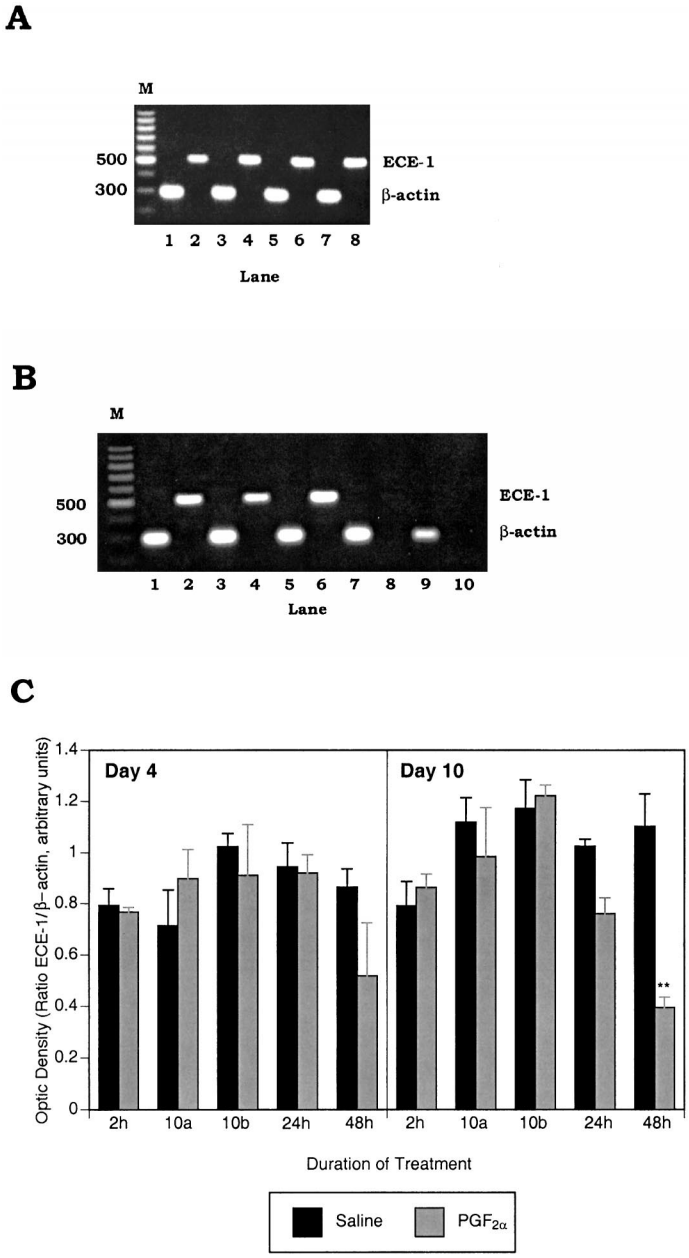

FIG. 1. Effects of PGF$_{2\alpha}$ on the amount of mRNA encoding ECE-1 during the early and midluteal phases of the bovine CL. Ten nanograms of total RNA, extracted from bovine CL, were subject to RT-PCR. The RT-PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed. The marker was a 100-bp marker, with the brightest band representing 500 bp. **A**) Representative photograph of the RT-PCR products obtained from total RNA from the Day 4 CL collected after 48 h. Lanes 1–4: samples from animals treated with saline; lanes 5–8: samples from animals treated with PGF$_{2\alpha}$. **B**) Photograph of the RT-PCR products of the Day 10 samples. The CL was collected at 48 h. Lanes 1–6: samples from animals treated with saline; lanes 7–10: animals treated with PGF$_{2\alpha}$. **C**) Amount of mRNA encoding ECE-1 during Day 4 and Day 10. Data are the mean ± SEM of the densitometric analyses of ECE-1 relative to β-actin mRNA. Treatments labeled as 10a received a single injection of saline or PGF$_{2\alpha}$; treatments labeled as 10b received two injections of saline or PGF$_{2\alpha}$. $^{**}P < 0.01$ versus saline control.

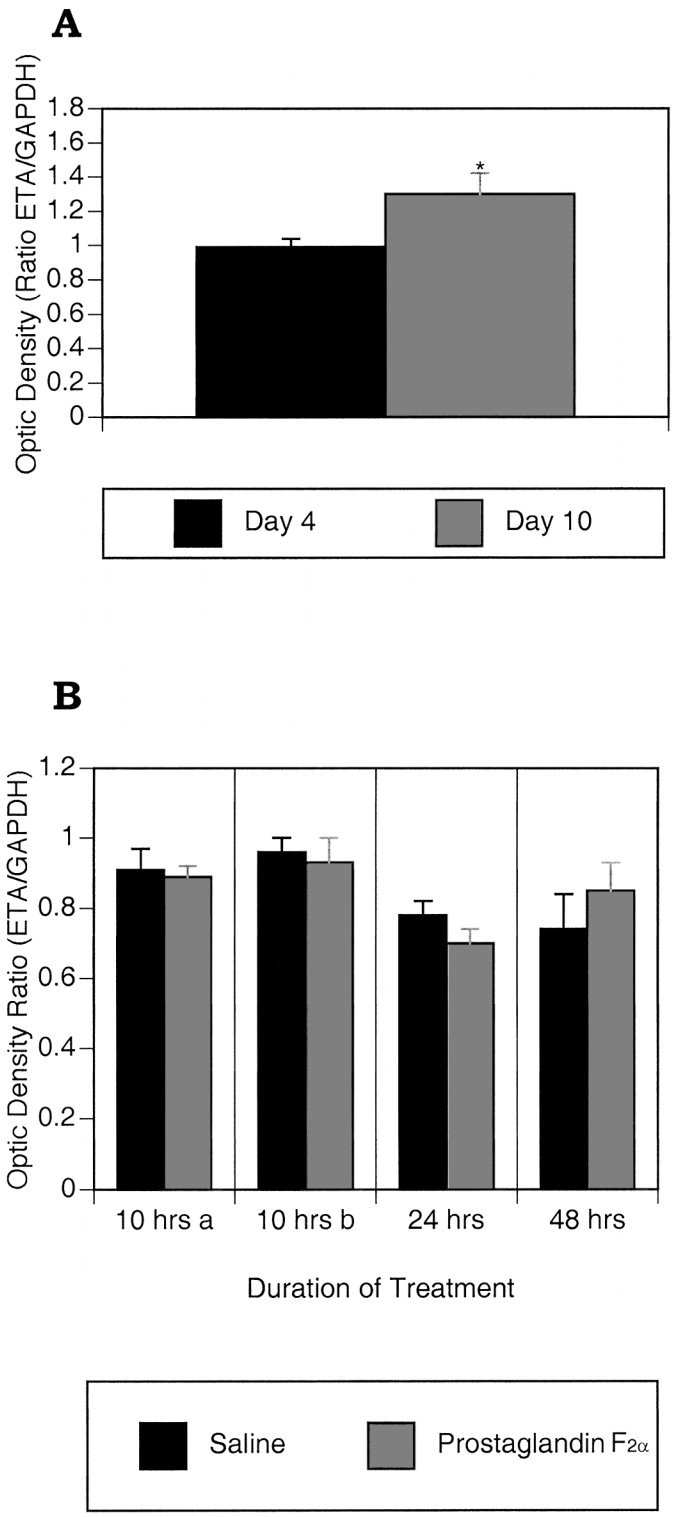

FIG. 2. Amount of mRNA encoding ET$_A$ during the early and midluteal phases of bovine CL and effects of PGF$_{2\alpha}$ administration during the midluteal phase. Data are the mean ± SEM of the densitometric analyses of ET$_A$ relative to GAPDH mRNA. **A**) Amount of mRNA encoding ET$_A$ during the early phase (Day 4) and midluteal phase (Day 10) of the bovine CL. $^{*}P < 0.05$. **B**) Effects of PGF$_{2\alpha}$ administration on the amount of mRNA encoding ET$_A$ during Day 10. Treatments labeled as 10 hrs a received a single injection of saline or PGF$_{2\alpha}$; treatments labeled as 10 hrs b received two injections of saline or PGF$_{2\alpha}$.

Effect of PGF$_{2\alpha}$ on Luteal ET$_A$ mRNA

Figure 2A shows that the amounts of mRNA encoding ET$_A$ were significantly higher in Day 10 than in Day 4 CL ($P < 0.05$). However, administration of PGF$_{2\alpha}$ did not affect the mRNA for ET$_A$ at any time period examined for

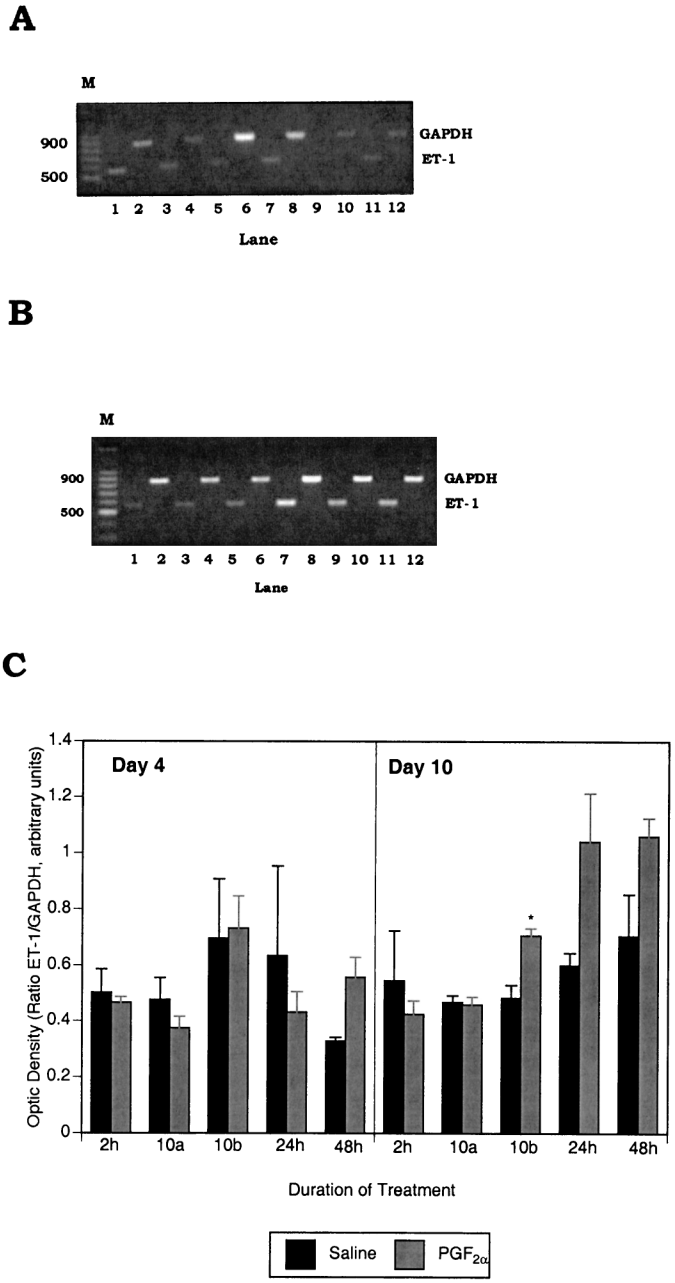

FIG. 3. Effects of $PGF_{2\alpha}$ on the amount of mRNA encoding ET-1 during the early and midluteal phases of the bovine CL. From bovine CL, 100 ng/μl of total RNA were extracted and subjected to RT-PCR. The RT-PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed. The marker was a 100-bp marker, with the brightest band representing 500 bp. **A**) Photograph of the RT-PCR products obtained from total RNA from the Day 4 CL treated with two injections of saline or $PGF_{2\alpha}$ and collected after 10 h. Lanes 1–6: samples from animals treated with saline; lanes 7–12: animals treated with $PGF_{2\alpha}$. **B**) Photograph of the RT-PCR products of the Day 10 samples. The CL was treated with two injections of saline or $PGF_{2\alpha}$ and collected after 10 h. Lanes 1–6: samples from animals treated with saline; lanes 7–12: animals treated with $PGF_{2\alpha}$. **C**) The amount of mRNA encoding ET-1 during Day 4 and Day 10. Data are the mean ± SEM of the densitometric analyses of ET-1 relative to GAPDH mRNA. Treatments labeled as 10a received a single injection of saline or $PGF_{2\alpha}$; treatments labeled as 10b received two injections of saline or $PGF_{2\alpha}$. $^*P < 0.05$ versus saline control.

the Day 10 (Fig. 2B) or Day 4 CL (data not shown). The amount of ET_A protein in the Day 4 or Day 10 CL was not affected by treatment with $PGF_{2\alpha}$.

Effect of $PGF_{2\alpha}$ on Luteal ET-1 mRNA

Figure 3A shows a representative photograph of the RT-PCR products obtained with the ET-1 primers from total RNA of Day 4 CL treated with two injections of saline or $PGF_{2\alpha}$ and collected after 10 h. Lanes 1–6 represent samples from animals treated with saline. Lanes 7–12 represent animals treated with $PGF_{2\alpha}$. Figure 3B shows a representative photograph of the RT-PCR products obtained with the ET-1 primers in the Day 10 CL as treated with two injections of saline or $PGF_{2\alpha}$ and collected after 10 h. Lanes 1–6 represent samples from animals treated with saline. Lanes 7–12 represent animals treated with $PGF_{2\alpha}$. Figure 3C shows that, in the early CL, $PGF_{2\alpha}$ treatment had no effect on the amount of mRNA encoding ET-1 at any time point examined ($P > 0.1$). During the midluteal phase of the cycle, a single injection of $PGF_{2\alpha}$ had no effect on the amount of mRNA encoding the ET-1 up to 10 h. In the midluteal CL collected after 10 h, the two injections of $PGF_{2\alpha}$ induced a statistically significant ($P < 0.05$), 55% increase in the amount of mRNA encoding ET-1. By 24 h, the amount of ET-1 mRNA was increased 66%, but this increase was not statistically significant. After 48 h, the amount of ET-1 mRNA was increased by approximately 54% ($P = 0.059$) from the saline control.

Effect of $PGF_{2\alpha}$ on Luteal ECE-1 Protein Concentration

In the early CL, $PGF_{2\alpha}$ treatment had no effect on the amount of ECE-1 protein at any time point examined ($P > 0.1$) (Fig. 4A). During the midluteal phase of the cycle, only the 48-h treatment with $PGF_{2\alpha}$ induced a statistically significant ($P > 0.01$), 12% reduction in the amount of ECE-1 protein (Fig. 4A). In the 24-h treatment group, $PGF_{2\alpha}$ had no effect on the amount of ECE-1 protein ($P > 0.9$) (Fig. 4A).

Effect of $PGF_{2\alpha}$ on Luteal ET_A Protein Concentration

Administration of $PGF_{2\alpha}$ did not affect the amount of ET_A protein at any time point examined for Day 10 (Fig. 4B) or Day 4 CL. Surprisingly, no statistical differences were observed in the amount of ET_A protein present in the Day 4 CL from the amount detected in the Day 10 CL (Fig. 4B).

Figure 5 shows representative Western blots corresponding to the 48-h treatment for Day 10 (Fig. 5A) and Day 4 (Fig. 5B) CL. Protein bands of 130, 57, and 43 kDa were immunodetected with the antibodies for ECE-1, ET_A, and actin, respectively.

DISCUSSION

In these studies, we have demonstrated a novel action of $PGF_{2\alpha}$ during the midluteal phase of the bovine CL: It regulates the amount of mRNA encoding ECE-1. This novel luteal action for $PGF_{2\alpha}$ appears to be inhibitory in nature. It was first observed after 24 h in CL of cows that received multiple injections of $PGF_{2\alpha}$. Importantly, this novel action of $PGF_{2\alpha}$ was cycle-phase specific, because it was not observed in the early CL. This finding supports and expands the work by Meidan and Levy [32], who reported the expression of ECE in the bovine CL.

This observation implies that a $PGF_{2\alpha}$-mediated up-reg-

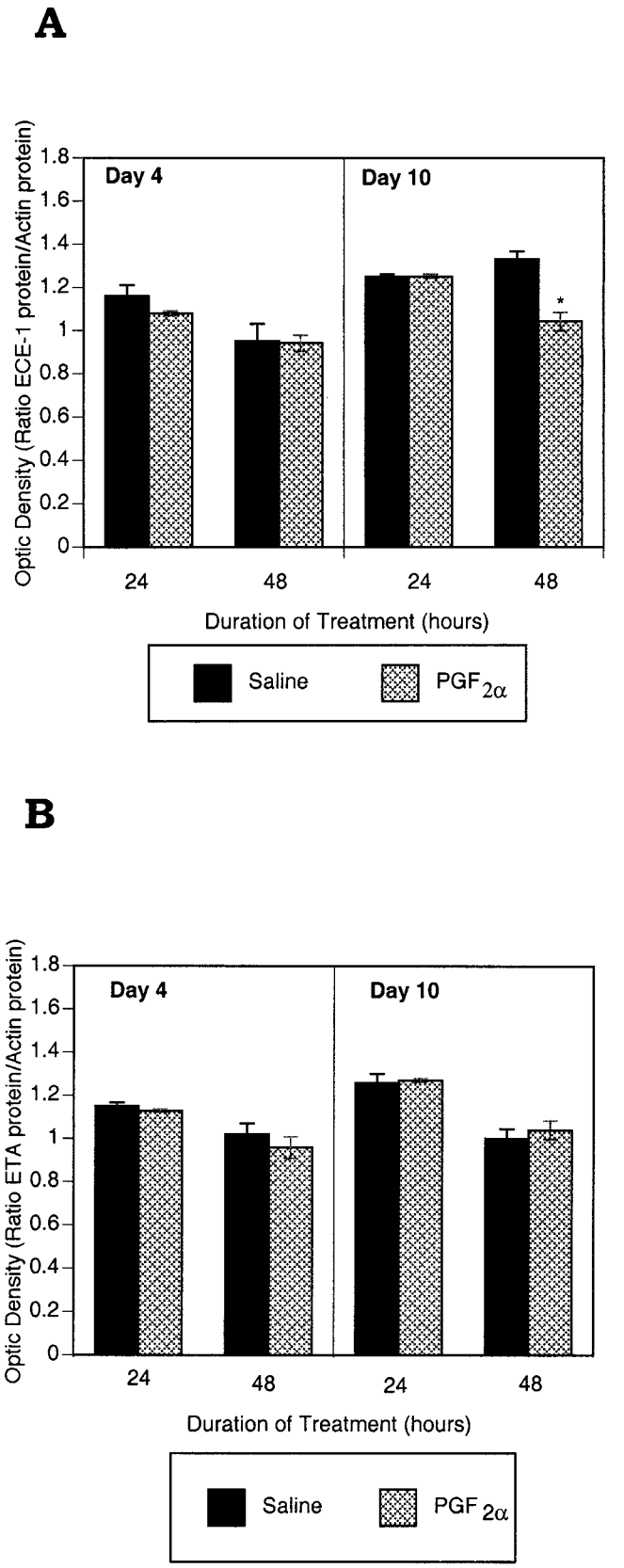

FIG. 4. Summary of the Western blot analyses quantified by scanning densitometry. The effects of saline and $PGF_{2\alpha}$ on the amount of ECE-1 and ET_A protein during the early (n = 15) and midluteal (n = 12) phases of the bovine CL at 24 and 48 h are presented. Data are the mean ± SEM of the densitometric analyses of ECE-1 or ET_A relative to actin protein. **A**) ECE-1 protein. $*P < 0.05$. **B**) ET_A protein.

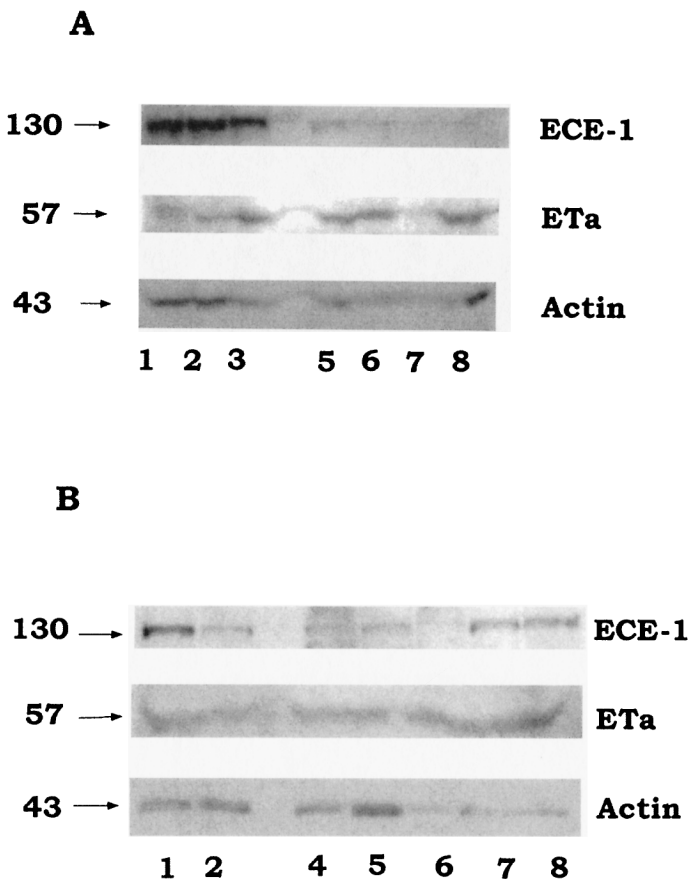

FIG. 5. Representative Western blots demonstrating the effects of $PGF_{2\alpha}$ on ECE-1 and ET_A in bovine CL. Thirty micrograms of protein samples were used in SDS-PAGE. Visualization of bands was achieved using an enhanced chemiluminescence detection system. The intensity of the signal corresponding to the ECE-1 and ET_A proteins were standardized by the corresponding intensity of the actin protein control. The sizes of the proteins are: ECE-1, 130 kDa; ET_A, 57 kDa; and actin, 43 kDa. **A**) Photograph of the Western blot analysis of ECE-1 and ET_A protein samples obtained from the Day 10 CL collected after 48 h. Lanes 1–3: samples from animals treated with saline; lanes 5–8: samples treated with $PGF_{2\alpha}$. **B**) Photograph of the Western blot analysis of ECE-1 and ET_A protein samples obtained from the Day 4 CL collected after 48 h. Lanes 1–2: samples treated with saline; lanes 4–8: samples treated with $PGF_{2\alpha}$.

ulation of the ECE-1 gene is not required for $PGF_{2\alpha}$ to up-regulate ET-1 during the midluteal phase. In fact, we observed a down-regulation of ECE-1 mRNA and protein by $PGF_{2\alpha}$. Evidently, the amount of ECE-1 mRNA and protein were not different between the Day 4 and Day 10 CL. Furthermore, $PGF_{2\alpha}$, in a phase-specific fashion, down-regulates the expression of this gene in the Day 10 CL. This phase-specific action of $PGF_{2\alpha}$ on the ECE-1 gene is unlikely to contribute to the complex mechanism responsible for the insensitivity of early CL to the luteolytic actions of $PGF_{2\alpha}$. A more likely candidate gene would be the phase-specific action of $PGF_{2\alpha}$ on ppET-1 mRNA, as observed in this study and reported by Levy et al. [21]. This later observation by Levy et al. [21], corroborated in the present study, supports the proposed participation of endothelin in mediating the luteolytic actions of $PGF_{2\alpha}$ in the CL.

The mechanism responsible for the resistance observed in the early CL to the luteolytic actions of $PGF_{2\alpha}$ is not fully understood, but several potential components have been implicated. Silva et al. [44] recently demonstrated that luteal prostaglandin dehydrogenase (PGDH) activity in the

CL of the ewe is greater on Day 3 than on Day 13 of the estrous cycle. The PGDH is responsible for converting $PGF_{2\alpha}$ to its inactive metabolite; therefore, its higher activity in the early CL could account for lower intraluteal concentrations of $PGF_{2\alpha}$ in the early CL, leading to its resistance. Tsai et al. [18] as well as Tsai and Wiltbank [45] have reported that a single injection of $PGF_{2\alpha}$ up-regulated mRNA encoding prostaglandin G/H synthase 2 (PGHS-2, COX 2) in the mid- and late-cycle ovine and bovine CL, but that it was not effective in animals on Day 4 of the estrous cycle. Therefore, these investigators proposed that secretion of $PGF_{2\alpha}$ by late-cycle CL amplified the luteolytic signal from the uterus in an autocrine/paracrine manner [45]. Because this amplification is not observed in the early CL, this could account for its resistance to the luteolytic actions on $PGF_{2\alpha}$. In contrast, Sayre et al. [43] found that repeated treatment with $PGF_{2\alpha}$ up-regulated PGHS-2 and $PGF_{2\alpha}$ synthase in Day 4 CL.

Levy et al. [21] have reported another possible cause for the resistance of early CL. These investigators have stated that administration of $PGF_{2\alpha}$ during the early bovine luteal phase does not alter expression of ET-1 or ET_A. In contrast, ET-1 and ET_A mRNA were markedly induced during the midluteal phase of the CL when exposed to a similar dose of $PGF_{2\alpha}$.

Although we detected a higher amount of mRNA encoding ET_A in Day 10 than in he Day 4 CL, this was not the case at the protein level. Furthermore, we failed to detect any regulatory effect by $PGF_{2\alpha}$ on this gene at the mRNA and protein level. This observation contrasts with that of Levy et al. [21], who found that $PGF_{2\alpha}$ administered during the midluteal phase was able to induce both ET-1 and ET_A. These investigators also reported that, during the early luteal phase, administration of $PGF_{2\alpha}$ did not alter the expression of the ET_A [21]. We examined CL at 2, 10, 24, and 48 h and multiple challenges with $PGF_{2\alpha}$, but we never observed a stimulatory effect on this gene by $PGF_{2\alpha}$ in the Day 4 or Day 10 bovine CL. The reason for this discrepancy is not clear. However, in the study by Levy et al. [21], the dairy cows used for the midluteal stage varied from Day 10 to Day 17. If their midluteal samples were closer to Day 17, the luteal tissue could possibly have developed greater $PGF_{2\alpha}$ sensitivity. Our observation would imply that the mRNA encoding ET_A is up-regulated from levels observed on Day 4 to those seen on Day 10 by $PGF_{2\alpha}$-independent mechanisms. However, as mentioned earlier, this was not translated into a greater amount of ET_A protein.

Although we never observed a stimulatory effect on the ET_A gene by $PGF_{2\alpha}$, we did not see any inhibitory effect, even after 48 h with multiple injections. Clearly, this gene is regulated very differently than the ECE-1 gene. The results of our studies indicate that if the ET system mediates the luteolytic action of $PGF_{2\alpha}$, an up-regulation of the ET system from the Day 4 to the Day 10 CL does not require an increased expression of the ET_A and ECE-1 genes by $PGF_{2\alpha}$. However, as argued above, an increased sensitivity of the CL for $PGF_{2\alpha}$ to up-regulate these components of the endothelin system possibly is reached later than the luteal stages examined here. If ET-1 participates in mediating the luteolytic actions of $PGF_{2\alpha}$, the gene expression of the ET_A and ECE-1 do not appear to be the limiting factors of the CL endothelin system. Our observation that the amount of mRNA encoding ECE-1 is down-regulated by $PGF_{2\alpha}$ in the Day 10, but not in the Day 4, CL may be a result of the increased ET-1 by $PGF_{2\alpha}$ at these developmental stages of the CL. A similar finding has been reported in the vascular system of the rat pulmonary endothelial cells, in which ET-1 inhibits ECE-1 expression [46].

Clearly, these actions of $PGF_{2\alpha}$ during the midluteal phase of the CL can be dissociated from its actions on P_4. First, a single injection of $PGF_{2\alpha}$ during the midluteal phase of the CL, which effectively reduced the plasma concentration of P_4, had no effect on the amount of mRNA encoding ECE-1 and ET-1 by 10 h. Second, multiple injections of $PGF_{2\alpha}$ in the early CL effectively reduced plasma levels of P_4 without any effect on mRNA encoding ECE-1 or ET-1. Unchanged mRNA concentrations for ECE-1 possibly were enough to maintain the necessary amount of bioactive ET-1. This is an issue that needs to be explored further in future studies. In fact, a reduction in mRNA encoding ECE-1 was detected after 24 h, but a reduction in the amount of ECE-1 protein was not detected until 48 h. It will be necessary to determine the effect of $PGF_{2\alpha}$ on the actual ECE-1 activity. Certainly, the amount of ppET-1 mRNA was increased by $PGF_{2\alpha}$ at a time when both ECE-1 mRNA and protein were reduced by the $PGF_{2\alpha}$ treatment. This increase in ppET-1 mRNA induced by $PGF_{2\alpha}$ also likely results in an increase in ET-1 peptide [47]. If so, then three scenarios can be envisioned. First, the observed reduction in ECE-1 mRNA possibly is not to a level at which the ECE-1 protein and activity would be reduced and become rate-limiting in the production of bioactive ET-1. Second, ECE-1 is not the enzyme responsible for generating bioactive ET-1 in the bovine CL. Third, an ECE-1 isoform different from the one that we have examined (i.e., ECE-1α) is compensating for the reduced ECE-1 mRNA and protein. In the human vascular system, evidence indicates that, by alternative splicing, the ECE-1 gene can generate several ECE-1 isoforms [48]. At this time, we cannot distinguish among these possible interpretations.

If ET-1 participates in mediating the luteolytic actions of $PGF_{2\alpha}$, then the results of our studies indicate that the ET-1 gene is the component of the endothelin system most likely to be involved with $PGF_{2\alpha}$ regulation. The ECE-1 and the ET_A appear to be constitutively expressed in the Day 4 and Day 10 CL.

In summary, we report in this study a novel action of $PGF_{2\alpha}$ during the midluteal phase of the bovine CL: It regulates the amount of mRNA encoding ECE-1. This novel luteal action for $PGF_{2\alpha}$ appeared to be inhibitory in nature. Importantly, this novel action of $PGF_{2\alpha}$ was cycle-phase specific, because it was not observed in the early CL. We suggest that if ET-1 participates in mediating the luteolytic actions of $PGF_{2\alpha}$, then the most likely component of the endothelin system in the bovine CL target for $PGF_{2\alpha}$ regulation is the ET-1 gene. Finally, an important practical implication of this observation is that it may be possible to target the ET-1 gene as an effective way to manipulate the luteolytic actions of $PGF_{2\alpha}$.

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