Effect of Intraluteal Injection of Endothelin Type A Receptor Antagonist on $PGF_{2\alpha}$-induced Luteolysis in the Cow

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Running head: ET-1 IN STRUCTURAL LUTEOLYSIS

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Abstract

Endothelin-1 (ET-1) is a luteolytic mediator in the bovine corpus luteum (CL), and its action appears to be via endothelin type A receptor (ETR-A). Thus, the aim of the present study was to determine the effect of ETR-A antagonist on $PGF_{2\alpha}$-induced luteolysis in the cow. Cows on days 10-12 of the estrous cycle were subjected to five intraluteal injections of the ETR-A antagonist LU 135252 in saline or only saline at -0.5, 2, 4, 6, and 8 h after $PGF_{2\alpha}$ administration (= 0 h). Serial luteal biopsies were conducted to determine the expression of mRNA in the luteal tissue. There were no significant differences in the decrease in plasma progesterone (P) concentrations and the mRNA expressions of steroidogenic acute regulatory protein and 3β-hydroxysteroid dehydrogenase/Δ^5,Δ^4-isomerase between the ETR-A antagonist-treated group and the control group. However, the start of the decline in CL volume and blood flow area surrounding the CL was delayed for almost two days in the ETR-A antagonist-treated group compared to the control group. The mRNA expression of preproET-1 and endothelin type B receptor increased in both groups, while the ETR-A mRNA remained unchanged. In addition, caspase-3 mRNA expression increased significantly at 24 h in the control group only and its level was higher than that of the ETR-A antagonist-treated group. Thus, the present study suggests that ET-1 regulates structural luteolysis via ETR-A by controlling blood vessel contraction in the CL of the cow.

Key words: Apoptosis, Blood Flow, Corpus Luteum, Cow, Endothelin-1

Introduction

The corpus luteum (CL) is a transient endocrine gland that secretes progesterone (P) to support pregnancy. If fertilization does not occur, the CL undergoes regression to induce the next ovulation. In ruminants, it is well known that the prostaglandin (PG) $F_{2\alpha}$ released from the endometrium is a primary luteolysin [1]. A pulsatile release of physiological $PGF_{2\alpha}$ on days 17-18 (estrus = day 0) or an injection of exogenous $PGF_{2\alpha}$ during the mid-luteal phase of the estrous cycle induces a drastic decrease in the plasma P concentration followed by a gradual decline in blood flow to the CL and a decrease in the CL tissue [2-4]. However, our previous studies have indicated that direct exposure of the microenvironment within the mid-CL to $PGF_{2\alpha}$ using an in vivo [5] and in vitro [6, 7] microdialysis system (MDS) did not inhibit, but rather stimulated P secretion from the CL. These observations suggest that $PGF_{2\alpha}$ may stimulate several luteolytic mediators, such as endothelin-1 (ET-1), angiotensin II (Ang II), and nitric oxide (NO), rather than direct action on luteal cells to inhibit P synthesis when it reaches the CL through the blood vessels [8-10].

Endothelin-1, a 21-amino acid peptide produced by endothelial cells, was first isolated from porcine vascular endothelial cells [11]. ET-1 is the most potent vasoconstrictor identified so far, and it exerts a wide spectrum of biological effects in different tissues. This peptide binds to two distinct subtypes of G protein-coupled receptors, termed endothelin type A receptor (ETR-A) and endothelin type B receptor (ETR-B) [12, 13]. ET-1 elicits vasoconstriction mostly via ETR-A [14, 15] and contrarily induces vasodilation via ETR-B [16, 17]. The ET system is expressed in the bovine CL throughout the estrous cycle, and preproET-1 mRNA and peptide expression increase to their highest levels during luteal regression [10, 18-20]. A number of studies using luteal slices [21], cultured luteal endothelial cells [10], and microdialyzed CL, in vivo [22] and in vitro [7] have indicated that $PGF_{2\alpha}$ stimulates biosynthesis of ET-1 in the CL. In addition, ET-1 and/or $PGF_{2\alpha}$ inhibit P secretion from the CL, and this inhibition was blocked by an ETR-A antagonist [21, 23-25]. Moreover, intraluteal administration of an ETR-A antagonist during the mid-luteal phase interrupted the luteolytic effect of $PGF_{2\alpha}$ in the ewe [24].

Thus, it is considered that ET-1 mediates the luteolytic action of $PGF_{2\alpha}$ via ETR-A in the bovine CL. However, the impact of in vivo ET-1 action in the cow via ETR-A during luteolysis has not

been fully elucidated. Therefore, we aimed to determine the effect of ETR-A antagonist on $PGF_{2\alpha}$-induced luteolysis in the cow. For this purpose, we injected ETR-A antagonist directly into the mid-CL during the early stage of $PGF_{2\alpha}$–induced luteolysis and observed the local effects on plasma P concentration, CL volume, and luteal blood flow area by color doppler ultrasonography, along with mRNA expression by trans-vaginal serial luteal biopsies.

Materials and Methods

Animals

The experiment was conducted at the Field Center of Animal Science and Agriculture, Obihiro University, and the experimental procedures complied with the Guidelines for the Care and Use of Agricultural Animals of Obihiro University. The animals had at least two estrous cycles of normal length (21 - 23 days) before being subjected to the experiment.

Experimented design

Twelve multiparous, nonlactating Holstein cows were used for this study (ETR-A antagonist-treated group, n = 7; control group, n = 5). Luteolysis was induced by intramuscular (i.m.) injection of 500 μg of $PGF_{2\alpha}$ analogue (cloprostenol, Estrumate®, Schering-Plough Animal Health K. K., Tokyo, Japan), and after 48 h, 100 μg of GnRH (Conceral®, Schering-Plough Animal Health K. K.) was injected to ensure ovulation. The day of estrus was designated as day 0. Before the experiment, ovaries were monitored by transrectal ultrasonography using an ultrasound scanner (Aloka SSD-5500, Mitaka, Tokyo, Japan) equipped with a 7.5 MHz convex transducer (Aloka UST-995-7.5) to determine that the CL had no cavity and that it was normal. On days 10-12 of the estrous cycle (estrus = day 0), 500 μg of $PGF_{2\alpha}$ analog was injected to induce luteolysis ($PGF_{2\alpha}$ i.m. = 0 h). Cows were subjected to five intraluteal injections of the ETR-A antagonist LU 135252 (Darusentan, Knoll, Ludwigshafen, Germany) in saline (50 mg/250 μl/shot; ETR-A antagonist-treated group) or saline only (vehicle; control group) at -0.5, 2, 4, 6, and 8 h after $PGF_{2\alpha}$ administration. Dose and frequency for the ETR-A antagonist were determined by preliminary experiments. Ultrasonographic examinations were carried out at -0.5, 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48, 72, and 96 h after $PGF_{2\alpha}$ administration. Blood samples were collected at each of these times and additionally at -2, -1.5, and -1 h to determine plasma P concentration. The plasma obtained was stored at -30 °C until analyzed. Serial luteal biopsies were collected at -0.5, 0.5, 4, 12, and 24 h after $PGF_{2\alpha}$ administration to determine the expression of mRNA in the CL. The time schedule of the present study is shown in Fig. 1.

Ultrasound scanning and intraluteal injection of ETR-A antagonist and serial luteal biopsies

The CL was examined by transrectal ultrasonography. During each ultrasonographic examination, the volume of the CL and blood flow area surrounding the CL was evaluated as described previously [26]. For intraluteal injection of the ETR-A antagonist, double structure needles, 18-gauge and 23-gauge, were used. Cows were encaged in a stall, and caudal epidural anesthesia was induced with 6 ml lidocaine hydrochloride (AstraZeneca, Xylocaine® 2 % (w/v), Osaka, Japan). A stainless steel handle containing a needle guide was mounted onto a 7.5 MHz convex array ultrasound transducer (Aloka UST-M15-21079). During ETR-A antagonist injection, the transducer face was applied to the wall of the vaginal fornix and the external needle was stuck into the ovary through the vaginal fornix. After confirming that the internal needle was located in the center of the CL, ETR-A antagonist or vehicle was administrated carefully. The injected agent was observed as a white shade on a monitor and was seen to diffuse within the CL. One ml disposable syringes were filled with either 250 µl ETR-A or saline and with 100 µl of saline (the dead volume of the customized needle). Trans-vaginal ultrasound-guided serial luteal biopsies were conducted as described by Tsai *et al*. [27]. For the serial luteal biopsies, 60 cm, 18-gauge biopsy needles (US Biopsy, SABD-1860-15-T, Franklin, IN 46131, USA) were used. In the serial luteal biopsies, the tissues were removed from the specimen notch, placed into a 1.5 ml microcentrifuge tube with 400 µl of TRIzol reagent (Gibco BRL, Gaithersburg, MD), homogenized immediately, and stored at -80 °C until analysis.

RNA extraction and reverse transcription (RT)

Total RNA was extracted from CL biopsy following the protocol of Chomczynski and Sacchi [28] using TRIzol reagent and was treated with DNase using a commercial kit (SV total RNA Isolation System: Promega Co., Madison, WI, USA). They were then frozen at -20 °C in THE RNA storage Solution (Ambion, Inc., Austin, TX, USA). Single-strand cDNA was reverse transcribed from total RNA (2 µg) using a 1st Strand cDNA Synthesis Kit for RT-PCR [AMV] (Roche Diagnostics, Indianapolis, IN, USA) and a random primer. The RT cycle consisted of 10 min annealing at 25 °C, 50 min cDNA synthesis at 42 °C, and 15 min inactivation at 72 °C.

Quantitative PCR

The mRNA expression of preproET-1, ETR-A, ETR-B, steroidogenic acute regulatory protein (StAR), 3β–hydroxysteroid dehydrogenase/Δ^5, Δ^4-isomerase (3β-HSD), caspase-3, and β-actin were quantified by real-time PCR with a LightCycler (Roche Diagnostics) using a commercial kit (LightCycler FastStart DNA Master SYBR Green I, Roche Diagnostics). The primers were designed using Primer-3 based on bovine sequences (Table 1). The amplification program consisted of an initial 15 min activation at 95 °C followed by 40 cycles of PCR steps (15 sec denaturation at 94 °C, 30 sec annealing at 60 °C and 20 sec extension at 72 °C). For quantification of the target genes, a series of standards was constructed by amplifying a fragment of DNA (450-500 bp) that contained the target sequence for real-time PCR (100-150 bp). The PCR products were subjected to electrophoresis, and the target band was cut out and purified using a DNA purification kit, SUPRECTM-01 (TaKaRa Bio. Inc., Otsu, Japan). Three to five stepwise-diluted DNA standards were included in every PCR run. The values were normalized using β-actin as the internal standard.

Hormone determination

The concentration of P in the plasma was determined in duplicate by enzyme immunoassay (EIA) after diethyl ether extraction [29]. The residue from each 200 µl blood sample was dissolved in 200 µl of assay buffer (40 mM PBS, 0.1 % BSA, pH 7.2), and the recovery rate was 90 %. The standard curve ranged from 0.05 to 50 ng/ml, and the effective dose $(ED)_{50}$ of the assay was 2.4 ng/ml. The intra- and interassay coefficients of variation (CVs) were 6.2 % and 9.3 %, respectively.

Statistical analysis

The time of $PGF_{2\alpha}$ analogue injection was defined as 0 h. The volume of the CL, blood flow area surrounding the CL, and the expression of mRNA were expressed as the percentage of the individual baselines (-0.5 h). All data were expressed as mean ± SEM. The data regarding the changes in each group were examined by repeated-measures ANOVA with time as the variable tested. The data for the same time points between the ETR-A antagonist-treated group and the control group were analyzed using the Students t-test followed by the F-test. Probabilities less than 5 % ($P < 0.05$) were considered significant.

Results

The effect of the ETR-A antagonist on plasma P concentration

The basal concentration of plasma P was 4.54 ± 0.21 ng/ml (mean ± SEM). The plasma P concentration decreased significantly in both the control and ETR-A antagonist-treated groups at 6 h after $PGF_{2\alpha}$ administration, and there were no differences in plasma P concentration between the two groups at any time point (Fig. 2A).

The effect of the ETR-A antagonist on CL volume and blood flow area surrounding the CL

The volume of the CL before treatment (= 100 %) was 7.90 ± 0.56 cm^3 (mean ± SEM). CL volume began to decrease significantly at 6 h in the control group, and at 48 h in the ETR-A antagonist-treated group (Fig. 2B). Blood flow area surrounding the CL increased acutely in both groups at 0.5-2 h and decreased at 4 h to the same levels observed at 0 h (Fig. 2C). Thereafter, blood flow area further decreased at 48 h compared to baseline in the ETR-A antagonist-treated group, whereas a similar further decrease occured at 8 h in the control group (Fig. 2C).

The effect of the ETR-A antagonist on the expression of mRNA

The changes in mRNA expression of preproET-1, ETR-A, ETR-B, StAR, 3β-HSD, and caspase-3 are given in Fig. 3 and 4. PreproET-1 mRNA expression increased significantly at 12 and 24 h in the ETR-A antagonist-treated group and at 24 h in the control group (Fig. 3A). PreproET-1 mRNA expression in the ETR-A antagonist-treated group was significantly higher at 12 h than in the control group. ETR-A mRNA expression showed no clear change in either group (Fig. 3B). The ETR-B mRNA expression increased significantly at 24 h in both groups (Fig. 3C). ETR-B mRNA expression in the ETR-A antagonist-treated group appeared to be higher than in the control group at 24 h ($P = 0.065$). StAR and 3β-HSD mRNA expressions significantly decreased at 4 h in the ETR-A antagonist-treated group and at 0.5 h in the control group (Fig. 4A,B). There were no differences between the groups in the StAR and 3β-HSD mRNA expressions at any time point. Caspase-3 mRNA expression increased significantly at 24 h in only the control group, and its levels were higher than those of the ETR-A antagonist-treated group

(Fig. 4C).

Discussion

The results of the present study demonstrated that intraluteal injection of the ETR-A antagonist delayed the reduction in CL volume and blood flow area surrounding the CL, but did not affect the decrease in plasma P during $PGF_{2\alpha}$-induced luteolysis in the cow.

A previous study showed that blood flow to the CL was gradually reduced after the onset of luteolysis [30]. Furthermore our recent study showed that the blood flow area surrounding the CL increased acutely at the early stage of $PGF_{2\alpha}$-induced luteolysis and spontaneous luteolysis followed by a decrease in plasma P and CL volume [26, 31]. These observations indicate that this acute increase in blood flow surrounding the CL may be one of the initial steps for starting the luteolytic cascade in the CL. In the present study, in spite of intraluteal injection of the ETR-A antagonist, blood flow surrounding the CL increased acutely at 0.5-2 h after $PGF_{2\alpha}$, and this was similar to the controls, suggesting that ETR-A is not involved in the increase in blood flow surrounding the CL.

The CL is one of the most highly vascularized organs [32], and there are large arterioles surrounding the CL that presumably possess smooth muscle cells, whereas the microcapillary vessels inside the CL contain lesser amounts of smooth muscle cells [33]. In general, ETR-A is mostly expressed on smooth muscle cells, ET-1 elicits an increase in intracellular Ca^{2+} and inositol 1,4,5- trisphosphate via ETR-A, and the blood vessel contracts by the action of the surrounding smooth muscle cells [12, 14, 15]. Therefore, there is the possibility that ET-1 also acts on the blood vessels in the periphery of the CL and mediates vasoconstriction. In the present study, the mRNA expression for preproET-1 increased after $PGF_{2\alpha}$ administration in both groups, confirming the concept that ET-1 mediates the luteolytic action of $PGF_{2\alpha}$ on luteolysis. In addition, reduction in blood flow area surrounding the CL and CL volume were delayed by almost two days by the ETR-A antagonist treatment during $PGF_{2\alpha}$-induced luteolysis. We hypothesized that the action of ET-1 via ETR-A was weakened so that the constriction of the blood vessels surrounding the CL was softened. This mechanism may ensure blood flow supply to the CL and antagonize the reduction in CL volume. In addition, ETR-B is mostly expressed in endothelial cells and ET-1 elicits an increase in synthesis of NO and prostacyclin via ETR-B that induces blood vessel dilation [13, 16, 17]. It is possible that most of the free ET-1 bound to ETR-B and the blood vessels surrounding

the CL were dilated. To clarify this hypothesis, further study should focus on the localization and affinity of ETR-A and ETR-B in the bovine CL. Our data showed that the expression of caspase-3 mRNA, apoptosis-related gene significantly increased at 24 h only in the control group, but not in the ETR-A antagonist-treated group suggesting that the occurrence of apoptosis in the CL might be antagonized in the ETR-A antagonist-treated group.

The plasma P concentration decreased significantly at 6 h after $PGF_{2\alpha}$ injection in animals injected with ETR-A antagonist and in the control group. To support this finding, the expressions of mRNA for StAR and 3β-HSD decreased time-dependently and similarly in both groups. However, a previous study has clearly shown that ET-1 directly inhibits P secretion via ETR-A in bovine luteal cells [21]. This inconsistency may be due to the in vivo and in vitro experimental models used. It has been suggested that the decrease in P secretion during luteolysis may be induced by several factors, such as ET-1, Ang II, and NO. Ang II is the predominant vasoconstrictive peptide, and it is also a possible mediators of the luteolytic action of $PGF_{2\alpha}$ in the bovine CL [34-36]. On the other hand, NO is a local vasodilator that inhibits P secretion in bovine luteal cells [37]. In the present study, although the function of ET-1 via ETR-A was weakened, other luteolytic mediators such as luteal Ang II and NO may be highly stimulated by a luteolytic dose of $PGF_{2\alpha}$ and result in a normal decrease in the plasma P concentration. In the rabbit, the intravenous injection of ETR-A/B antagonist did not have any effect on the decrease of P secretion during $PGF_{2\alpha}$-induced luteolysis, further suggesting that other factors may compensate for the action of ET-1 to mediate the luteolytic cascade [38].

Hinckley and Milvae [24] reported that an intraluteal injection of ETR-A antagonist during $PGF_{2\alpha}$-induced luteolysis resulted in delay of functional luteolysis in the ewe. The reason for the discrepancy between the ewe and the cow is not clear. In the ewe, complete luteolysis was induced by an injection of ET-1 followed by a subluteolytic dose of $PGF_{2\alpha}$, but plasma P concentration only decreased to the sub-basal levels in the cow [39]. In addition, a recent study using the rabbit model showed that ET-1 alone could induce luteolysis, and this action was completely blocked by injection of ETR-A/B antagonist [38]. Therefore, there could be a definite difference between species in the degree of contribution of the ET-system in luteolysis.

We previously showed that changes in the cross-sectional area of the CL, as determined by

ultrasonography, were highly correlated with plasma P concentration [26]. However, in the present study, there was a considerable time difference (almost two days) between the start of the decrease in P secretion and in CL size in the ETR-A antagonist-treated group. Juengel *et al*. [40] reported that a low dose (3 mg/ 60 kg BW) of $PGF_{2\alpha}$ caused a decrease in P secretion without causing luteolysis, whereas a high dose (10 and 30 mg/ 60 kg BW) of $PGF_{2\alpha}$ caused a decrease in P secretion and luteolysis. It is therefore likely that functional luteolysis and structural luteolysis proceed in parallel during normal luteolysis, but these phenomena are controlled, at least in part, by different mechanisms.

In conclusion, the overall results suggest that ET-1 regulates structural luteolysis via ETR-A by controlling blood vessel function of the CL in the cow.

Acknowledgments

The authors thank Dr. K. Okuda, Okayama University, Japan, for P antiserum; Knoll GmbH, Germany, for the ETR-A antagonist; and Schering-Plough Animal Health K. K., Tokyo, Japan, for Estrumate® and Conceral®. This study was supported by a Grant-in–Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and the 21st Century COE program (A-1) of the Ministry of Education, Culture, Sports, Science and Technology of Japan. K.S. is supported by JSPS Research Fellowships for Young Scientists.

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Figure Legends

Fig. 1 Time schedule of the present in vivo experiment. The time of $PGF_{2\alpha}$ analogue injection was defined as 0 h, and we conducted ultrasonographic examination and blood sampling at all time points.

Fig. 2 Effects of intraluteal injection of the ETR-A antagonist on (A) plasma P concentration, (B) CL volume, and (C) blood flow area surrounding the CL. Data are shown as the mean ± SEM for each time point. The data for CL volume and blood flow area are expressed as the percentage of the respective baselines at -0.5 h (CL volume 100 % = 7.90 ± 0.56 cm^3). Symbols (* for the ETR-A antagonist-treated group and # for the control group) indicate significantly different values compared to the respective baseline values ($P < 0.05$).

Fig. 3 Effects of intraluteal injection of the ETR-A antagonist on the intraluteal mRNA expression of (A) preproET-1, (B) ETR-A, and (C) ETR-B. All data are expressed as the percentage of the individual baselines at -0.5 h and are shown as the mean ± SEM for each time point. Symbols (* for the ETR-A antagonist-treated group and # for the control group) indicate significantly different values compared to the respective baseline values ($P < 0.05$). Letters (a, b) donate differences between the ETR-A antagonist-treated group and control group at the same time point ($P < 0.05$).

Fig. 4 Effects of intraluteal injection of the ETR-A antagonist on intraluteal mRNA expression of (A) steroidogenic acute regulatory protein (StAR), (B) 3β–hydroxysteroid dehydrogenase/Δ^5, Δ^4-isomerase (3β-HSD), and (C) caspase-3. All data are expressed as the percentage of the individual baselines at -0.5 h and are shown as the mean ± SEM for each time point. Symbols (* for the ETR-A antagonist-treated group and # for the control group) indicate significantly different values compared to the respective baseline values ($P < 0.05$). Letters (a, b) donate differences between the ETR-A antagonist-treated group and control group at the same time point ($P < 0.05$).

Figure 1.

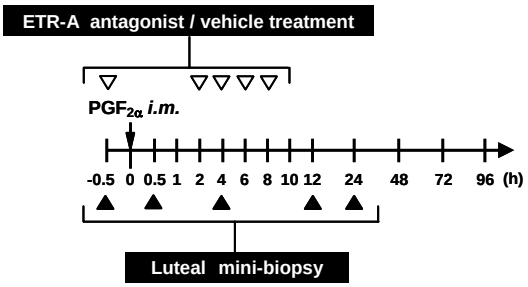
ETR-A antagonist / vehicle treatment
PGF2α i.m.
-0.5 0 0.5 1 2 4 6 8 10 12 24 48 72 96 (h)
Luteal mini-biopsy

Figure 2.

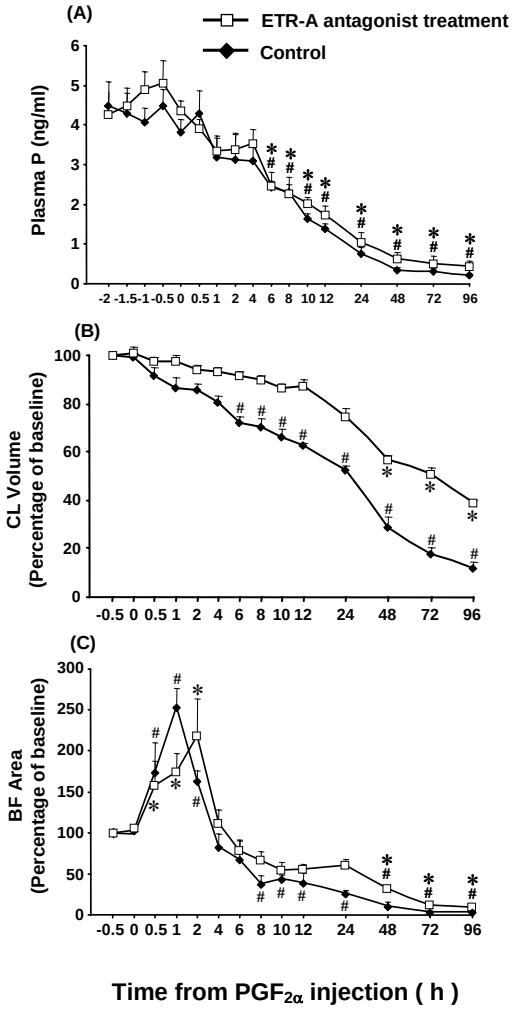
(A)
ETR-A antagonist treatment
Control
Plasma P (ng/ml)
(B)
CL Volume
(Percentage of baseline)
(C)
BF Area
(Percentage of baseline)
-2 -1.5 -1 -0.5 0 0.5 1 2 4 6 8 10 12 24 48 72 96
Time from PGF2α injection (h)

Figure 3

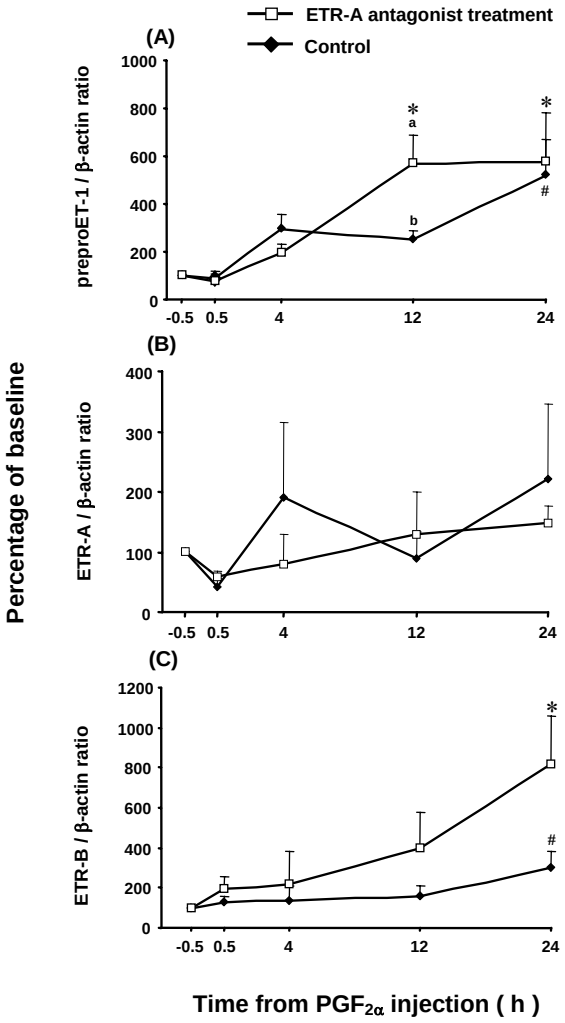
ETR-A antagonist treatment
Control
(A)
preproET-1 / β-actin ratio
1000
800
600
400
200
0
-0.5 0.5 4 12 24
*
a
b
#
(B)
ETR-A / β-actin ratio
400
300
200
100
0
-0.5 0.5 4 12 24
(C)
ETR-B / β-actin ratio
1200
1000
800
600
400
200
0
-0.5 0.5 4 12 24
*
#
Percentage of baseline
Time from PGF2α injection (h)

Figure 4.

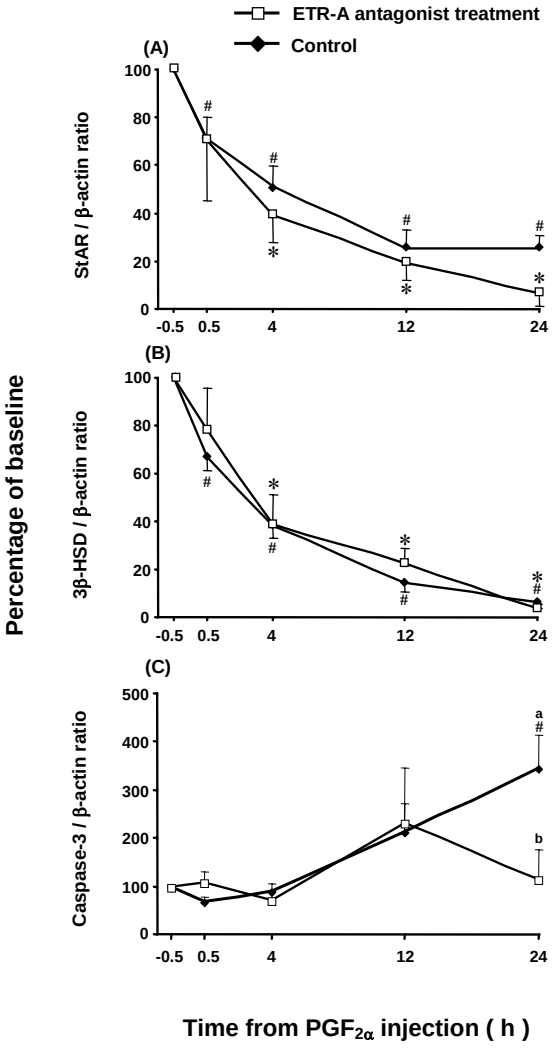
ETR-A antagonist treatment
Control
(A)
StAR / β-actin ratio
(B)
3β-HSD / β-actin ratio
(C)
Caspase-3 / β-actin ratio
Percentage of baseline
Time from PGF2α injection (h)
-0.5
0.5
4
12
24
0
20
40
60
80
100
200
300
400
500
#
*
a
b

Table 1. Primer sequence, resulting fragment size of preproendothelin-1 (ET-1), endothelin type A receptor (ETR-A), endothelin type B receptor (ETR-B), steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase/Δ^5, Δ^4-isomerase (3β-HSD), caspase-3, and β-actin as a internal standard.

Target	Sequence of nucleotide[a]	Fragment Size (bp)
ET-1	For 5'-CAAATGCATCCTGCCTGGTC-3' Rev 5'-ATTGCCACCCCCATAGAGGA-3'	160
ETR-A	For 5'-GCATCCAGTGGAAGAACCAT-3' Rev 5'-AACCAGTCAACCCTTCAACG-3'	227
ETR-B	For 5'-GCTCCATCCCACTCAGAAAA-3' Rev 5'-GCCAACACAGAGCAAAGACA-3'	242
StAR	For 5'-GTGGATTTTGCCAATCACCT-3' Rev 5'-TTATTGAAAACGTGCCACCA-3'	203
3β-HSD	For5'-TCCACACCAGCACCATAGAA-3' Rev 5'-AAGGTGCCACCATTTTTCAG-3'	178
caspase-3	For 5'-AAGCCATGGTGAAGAAGGAA-3' Rev 5'-CCTCAGCACCACTGTCTGTC-3'	182
β-actin	For 5'-CCAAGGCCAACCGTGAGAAGAT-3' Rev 5'-CCACGTTCCGTGAGGATCTTCA-3'	256

[a]For, forward; Rev, reverse.