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# Effects of prostaglandin $F_{2\alpha}$ on angiogenic and steroidogenic pathways in the bovine corpus luteum may depend on its route of administration

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## ABSTRACT

Prostaglandin (PG) $F_{2\alpha}$ and its analogs ($aPGF_{2\alpha}$) are used to induce regression of the corpus luteum (CL); their administration during the middle stage of the estrous cycle causes luteolysis in cattle. However, the bovine CL is resistant to the luteolytic actions of $aPGF_{2\alpha}$ in the early stage of the estrous cycle. The mechanisms underlying this differential luteal sensitivity, as well as acquisition of luteolytic sensitivity by the CL, are still not fully understood. Therefore, to characterize possible differences in response to $aPGF_{2\alpha}$ administration, we aimed to determine changes in expression of genes related to (1) angiogenesis—fibroblast growth factor 2 (FGF2), fibroblast growth factor receptor 1 (FGFR1), fibroblast growth factor receptor 2 (FGFR2), vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor receptor 1 (VEGFR1), vascular endothelial growth factor receptor 2 (VEGFR2); and (2) steroidogenesis—steroidogenic acute regulatory protein (STAR), cytochrome P450 family 11 subfamily A member 1 (P450scc), and hydroxy-delta-5-steroid dehydrogenase, 3 β- and steroid delta-isomerase 1 (HSD3B) in early- and middle-stage CL that accompany local (intra-CL) versus systemic (i.m.) $aPGF_{2\alpha}$ injection. Cows at d 4 (early stage) or d 10 (middle stage) of the estrous cycle were treated as follows: (1) systemic saline injection, (2) systemic $aPGF_{2\alpha}$ injection (25 mg), (3) local saline injection, and (4) local $aPGF_{2\alpha}$ injection (2.5 mg). Progesterone ($P_4$) concentration was measured in jugular vein blood samples during the entire set of experiments. After 4 h of treatment, CL were collected by ovariectomy, and mRNA and protein expression levels were determined by reverse transcription quantitative-PCR and Western blotting, respectively. Local and systemic $aPGF_{2\alpha}$ injections upregulated FGF2 expression but decreased expression of VEGFA in both CL stages. Both $aPGF_{2\alpha}$ injections increased the expression of *STAR* in early-stage CL, but downregulated it in middle-stage CL. In the early-stage CL, local administration of $aPGF_{2\alpha}$ upregulated *HSD3B*, whereas systemic injection decreased its mRNA expression in early- and middle-stage CL. Moreover, we observed a decrease in the $P_4$ level earlier after local $aPGF_{2\alpha}$ injection than after systemic administration. These results indicate that $aPGF_{2\alpha}$ acting locally may play a luteotrophic role in early-stage CL. The systemic effect of $aPGF_{2\alpha}$ on the mRNA expression of genes participating in steroidogenesis seems to be more substantial than its local effect in middle-stage CL.

**Key words:** cow, corpus luteum, prostaglandin $F_{2\alpha}$

## INTRODUCTION

Hormonal treatment using either naturally produced $PGF_{2\alpha}$ or $PGF_{2\alpha}$ analogs (**$aPGF_{2\alpha}$**) to manipulate the estrous cycle is commonly used in dairy cow breeding (Ahuja et al., 2005). Artificial shortening of the estrous cycle after $aPGF_{2\alpha}$ treatment is likely to influence follicle selection, subsequent ovulation, or the development of a functional corpus luteum (**CL**) in dairy cattle (Cuervo-Arango et al., 2011). It is well known that if pregnancy in cows is not established, $PGF_{2\alpha}$ is produced by uterine glands at the end of the luteal phase, which triggers luteolysis and initiates a new reproductive cycle (McCracken et al., 1999; Schams and Berisha, 2004; Ginther et al., 2009). Luteolysis can be induced pharmacologically in cattle during the middle stage (CL sensitivity to $PGF_{2\alpha}$; McCracken et al., 1999; Wenzinger and Bleul, 2012), but the newly formed CL is resistant to luteolysis induced by exogenous $PGF_{2\alpha}$ until d 5 of the estrous cycle (Pursley et al., 1995; Tsai and Wiltbank, 1998; Levy et al., 2000). However, the mechanisms underlying these differential $PGF_{2\alpha}$ effects are still not fully understood.

Development of the bovine CL is concomitant with intensive angiogenesis, which is crucial for its steroidogenic activity (Shirasuna et al., 2012; Miyamoto

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et al., 2013; Skarzynski et al., 2013). Several factors are involved in the regulation of ovarian angiogenesis and vascular function, such as fibroblast growth factor 2 (**FGF2**), which interacts with 4 signaling tyrosine kinase FGF receptors (**FGFR-1** to **FGFR-4**), and the vascular endothelial growth factor A (**VEGFA**) system, acting through its 2 tyrosine kinase receptors: vascular endothelial growth factor receptor 1 (**VEGFR1**) and vascular endothelial growth factor receptor 2 (**VEGFR2**; Berisha et al., 2000; Yamashita et al., 2008; Woad et al., 2009). The pathways mediating changes in progesterone ($\mathbf{P_4}$) production during the estrous cycle operate through regulation of the genes encoding steroidogenic enzymes: steroidogenic acute regulatory protein (*STAR*), cytochrome P450 Family 11 Subfamily A Member 1 (*P450scc*), and hydroxy-delta-5-steroid dehydrogenase, 3 β- and steroid delta-isomerase 1 (*HSD3B*; Hasegawa et al., 2019).

Previous reports have shown that $PGF_{2\alpha}$ differentially regulates mechanisms related to bovine CL development, maintenance, and regression in a cycle stage-dependent manner (Tsai and Wiltbank, 1998; Goravanahally et al., 2009; Mondal et al., 2011; Atli et al., 2012). Moreover, the effects of $PGF_{2\alpha}$ on bovine luteal steroidogenic cells may depend on its local, direct (autocrine/paracrine mode of action) effects or indirect effects, including several regulatory mechanisms in the female reproductive tract (e.g., endocrine action, blood flow regulation, involvement of the immune system; Pate, 1995; Korzekwa et al., 2006; Acosta et al., 2009).

We hypothesize that differences in the expression of genes related to growth factors involved in the development of blood vessels, cell proliferation, and steroidogenesis in the bovine CL may depend upon intra-CL (local) versus i.m. (systemic) administration of $aPGF_{2\alpha}$. Therefore, the goal of this study was to determine the effects of intra-CL versus i.m. $aPGF_{2\alpha}$ administration on systemic $P_4$ concentrations and the expression of factors related to (1) angiogenesis (FGF2, VEGFA, and their receptors) and (2) steroidogenesis (STAR, P450scc, and HSD3B) in early-stage (d 4 of the estrous cycle) and middle-stage (d 10 of the estrous cycle) CL at 4 h after $aPGF_{2\alpha}$ injections.

## MATERIALS AND METHODS

### Animals and Treatments

Our in vivo study was conducted in concordance with the appropriate guidelines: the EU Directive of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes (22 September 2010; no 2010/63/EU), Polish Parliament Act on Animal Protection (21 August 1997, Dz.U. 1997 No 111 poz. 724) with further updates; the Polish Parliament Act on the Protection of Animals Used for Scientific or Educational Purposes (15 January 2015, Dz.U. 2015 pos. 266). All animal procedures were reviewed and accepted following the guidelines of the Local Ethics Committee for Experiments on Animals in Olsztyn, Poland (approval no 23/2012/N).

In the present study, 48 healthy, cycling Polish Holstein-Friesian cows from a local commercial dairy farm ("Wolka Szlachecka," Jeziorany, Poland) were used. This study was conducted from July 2015 to December 2015. Cows were bred by AI with a standard, routine protocol. The farm is monitored by trained veterinary and nutrition consultants and was free of bovine herpesvirus 1 (BHV1), bovine viral diarrhea (BVD)/mucosal disease (MD), tuberculosis, and enzootic bovine leukosis (EBL). The experiment was performed in a group of nonpregnant cows (≥3 lactations, BCS = 3.5) that were considered for culling because of their low milk production. The experimental cows were housed in an indoor facility, were milked on a 12-h cycle, and fed a TMR to meet the nutritional requirements of milking cows (15–20 L/d) with ad libitum access to water and a salt-based mineral supplement. Before cows were enrolled in the experiment, an experienced veterinarian confirmed the absence of reproductive tract disorders by an ultrasonographic visualization per rectum using a 7.5-MHz linear array transducer (MyLab 30VET Gold Color Doppler Diagnostic Ultrasound System, ESOATE Pie Medica, Genoa, Italy). Moreover, all experimental cows underwent a general clinical examination in which rectal temperature, general attitude, and respiratory and heart rates were determined (data not shown). The estrous cycle was synchronized in all cows by 2 injections of $aPGF_{2\alpha}$ (25 mg of dinoprost, 5 mg/mL, Dinolytic, Zoetis Polska, Warszawa, Poland) 11 d apart, as reported previously (Skarzynski et al., 2009). Follicular development and structural changes of the CL during the entire estrous cycle were monitored using transrectal ultrasonography, and visible signs of estrus (i.e., vaginal mucus and standing behavior) were taken as its confirmation. The onset of estrus was considered d 0 of the estrous cycle. Additionally, the stage of estrous cycle was established by $P_4$ concentrations in blood plasma samples collected from the coccygeal vessels using RIA. The concentration of $P_4$ was 0.38 ± 0.09 ng/mL (mean ± SEM) in blood samples collected during estrus (d 0 of the estrous cycle).

### Intra-CL Injection and Ovary Collection

Each cow was treated with xylazine (i.m. 25–30 mg/animal; Xylavet 2%, ScanVet, Gniezno, Poland) followed by insertion of a polyvinyl catheter (outside

diameter = 2.1; inside diameter = 1.6 mm; Tomel Sp, Tomaszow Mazowiecki, Poland) into the jugular vein for frequent blood sample collections, as described previously by Skarzynski et al. (2003).

Cows were anesthetized via an epidural block using 4 mL of 2% procaine hydrochloride (Polocainum Hydrochloricum, Biowet Drwalew, Drwalew, Poland). Then, intra-CL injections were administered under ultrasound guidance through a sterile 1.25 × 50 mm (2-inch 18-gauge) ovum pick-up disposable veterinary injection needle (Bovivet, Poznan, Poland). The transducer and needle guide were coated with a sterile lubricant (Medicum, Lodz, Poland) and positioned within the vagina. The ovary bearing the CL was positioned via the rectum to visualize it. The needle was then passed through the vaginal wall, and $aPGF_{2\alpha}$ was injected directly into the CL. The injected agent was observed as a white shade on the monitor and was seen to diffuse within the CL.

Ovaries with CL were removed via the vagina by ovariectomy using a Hauptner's efeminator (Hauptner & Herberholz GmbH & Co. KG, Solingen, Germany). Ovary collection was described previously by Piotrowska et al. (2006).

### Experimental Design

The cows were separated into 2 cohorts based on the phase of the estrous cycle: group I (early stage; n = 24) and group II (middle stage; n = 24). On d 3 (group I) and d 9 (group II), a polyvinyl catheter was inserted into the jugular vein for frequent blood sample collection. Afterward, the cows at d 4 (group I) or d 10 (group II) were treated as follows: (1) i.m. (systemic) saline injection (control; n = 6); (2) i.m. (systemic) $aPGF_{2\alpha}$ injection (25 mg of dinoprost; n = 6); (3) intra-CL (local) saline injection (control; n = 6); and (4) intra-CL (local) $aPGF_{2\alpha}$ injection (2.5 mg of dinoprost; n = 6). To estimate the required dose of direct injection of $aPGF_{2\alpha}$ causing luteolysis, a dose of 2.5 mg of $aPGF_{2\alpha}$ was chosen. In a preliminary study, 3 different doses of $aPGF_{2\alpha}$ (1.25, 2.5, and 5 mg) were injected intra-CL to confirm the luteolytic effect of the $PGF_{2\alpha}$ on the bovine CL (data not published). The time of saline solution, intra-CL, or i.m. $aPGF_{2\alpha}$ injections was defined as 0 h. The CL were collected by ovariectomy 4 h after each treatment.

### Blood Collection and Plasma Progesterone Determination

Blood was aspirated from the jugular vein at −2, −1, 0, 0.25, 0.5, 0.75, 1, 2, and 4 h of the experiment into sterile 10-mL tubes containing 100 μL of 0.3 *M* EDTA and 1% acetylsalicylic acid, pH 7.4. After centrifugation (2,000 × *g*, 10 min at 4°C), the plasma was stored at −20°C for determination of $P_4$ concentration.

### Corpora Lutea Collection

Two equal parts of CL tissue were divided as follows: one part was placed into a 1.5-mL microcentrifuge tube containing 1 mL of Trizol reagent (15596-026, Invitrogen, Carlsbad, CA) and the second part was placed in an empty tube, immediately homogenized, and then stored at −80°C. The mRNA and protein expression of angiogenesis- and steroidogenesis-related factors in CL tissues was measured by reverse transcription quantitative (**RT-q**)PCR and Western blotting, respectively.

### RNA Extraction and cDNA Production

Total RNA was extracted from CL tissues using the Total RNA Prep Plus Kit (031-50; A&A Biotechnology, Gdansk, Poland) following the manufacturer's guidelines. The content and purity of RNA were estimated using the NanoDrop 1000 (ND-1000, Thermo Fisher Scientific, Wilmington, DE). The absorbance ratio (260/280 nm) for all samples was ~2.0, and the ratio at 260/230 nm ranged between 1.8 and 2.2. Then, 1 μg of RNA was reverse-transcribed into cDNA using a QuantiTect Reverse Transcription Kit (205311; Qiagen, Duesseldorf, Germany) following the manufacturer's guidelines. The cDNA was stored at −20°C until real-time RT-qPCR was carried out.

### Real-Time RT-qPCR

Real-time RT-qPCR assays were performed in an ABI 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green PCR master mix (Applied Biosystems). Primer sequences to determine the mRNA abundance of *FGF2*, *FGFR1*, *FGFR2*, *VEGFA*, *VEGFR1*, *VEGFR2*, *STAR*, *P450scc*, *HSD3B*, *GAPDH*, and *ACTB* were designed in Primer 3 (Koressaar and Remm, 2007; Untergasser et al., 2012). The housekeeping genes were chosen based on previous scientific reports: *GAPDH* (Hojoet al., 2016; Korzekwa et al., 2016), *ACTB* (Shirasuna et al., 2010; Herzog et al., 2012). In the present work, NormFinder (Andersen et al., 2004) analysis found *ACTB* to be the most stable gene. The primer sequences, GenBank accession numbers, and product sizes are presented in Table 1. Data were analyzed using the method described by Zhao and Fernald (2005). Gene expression data were expressed relative to the best housekeeping gene (*ACTB*) and are presented in arbitrary units. All primers were synthe-

**Table 1.** Sequences for primers, product size, and accession numbers for genes

| Gene | Primer sequence (5′-3′; F, forward; R, reverse) | | PCR product (bp) | Accession no. |
|---|---|---|---|---|
| *ACTB* | F | CCAAGGCCAACCGTGAGAAGAT | 256 | K00622 |
| | R | CCACGTTCCGTGAGGATCTTCA | | |
| *GAPDH* | F | CACCCTCAAGATTGTCAGCA | 103 | BC102589 |
| | R | GGTCATAAGTCCCTCCACGA | | |
| *STAR* | F | GGTGGTGGCACGTTTTCAAT | 79 | Y17259.1 |
| | R | CCTTGTCCGCATTCTCTTGG | | |
| *P450scc* | F | CAGCATATCGGTGACGTGGA | 139 | K02130.1 |
| | R | GGCCACCAGAACCATGAAAA | | |
| *HSD3B* | F | CTAATGGGTGGGCTCTGAAA | 473 | NM_174343 |
| | R | CACGCTGTTGGAAAGAGTCA | | |
| *FGF2* | F | GAGAAGAGCGACCCTCACA | 278 | NM_002006.3 |
| | R | TAGCTTTCTGCCCAGGTCC | | |
| *FGFR1* | F | CCGAGGCATGGAGTATCTTG | 158 | AJ004952 |
| | R | GGCCGTTGGTTGTCTTTTTA | | |
| *FGFR2* | F | AGCTCCTCCATGAACTCCAA | 214 | Z68150 |
| | R | CCTTGTCAATTCCCACTGCT | | |
| *VEGFA* | F | AGATCGAGTACATCTTCAAGCCATC | 66 | NM_174216 |
| | R | CGTCATTGCAGCAGCCC | | |
| *VEGFR1* | F | GAAGGACGGGATGAGGATGC | 186 | X94263 |
| | R | ATGGCGTTGAGCGGAATGTA | | |
| *VEGFR2* | F | TGGCCCAACAATCAGAGCAG | 154 | X94298 |
| | R | GAACGGAGCCCATGTCAGTG | | |

sized by Sigma (Custom Oligos Sigma-Aldrich, Madison, WI). The total reaction volume of 20 μL consisted of 10 μL of SYBR Green PCR master mix, 2 μL of forward and reverse primers each (250 n*M*), and 1 μL of cDNA (20 ng/μL). Real-time RT-qPCR was performed using the following settings: initial denaturation (10 min at 95°C), followed by 45 cycles of denaturation (15 s at 95°C) and annealing (1 min at 60°C). Melting curves were generated by stepwise increases in temperature from 60°C to 95°C after each PCR reaction to ensure single product amplification. Specificity of the product was confirmed by electrophoresis on 2% agarose gel.

### Western Immunoblotting

Protein expression levels for FGF2, FGFR1, FGFR2, VEGFA, VEGFR1, VEGFR2, STAR, P450scc, HSD3B, and GAPDH in the tissues were determined by Western blotting as previously described (Hojo et al., 2016). Specific antibodies are described in detail in Table 2. Protocols for overnight incubation were used following dilution of each antibody (Table 2) at 4°C. Subsequently, membranes were incubated with a 1:20,000 dilution of secondary polyclonal anti-goat IgG (sc-2347; Santa Cruz Biotechnology., Santa Cruz, CA), anti-rabbit IgG or anti-mouse IgG alkaline phosphatase-conjugated antibodies (S3687, S3562; Sigma Chemical Co., St. Louis, MO) for 1.5 h at room temperature. The immune complexes were detected using the alkaline phosphatase visualization procedure. The intensity of immunological reactions was evaluated by measuring the optical density area of each sample with computerized densitometry via NIH Image (National Institutes of Health, Bethesda, MD). Representative Western

**Table 2.** Specific antibodies used for Western immunoblotting

| Antibody | Clone | Biological source | Commercial source[1] | Dilution |
|---|---|---|---|---|
| Anti-GAPDH | Monoclonal | Mouse | Sigma, G8795 | 1:10,000 |
| Anti-STAR | Polyclonal | Rabbit | Abcam, ab96637 | 1:1,000 |
| Anti-P450scc | Polyclonal | Goat | Santa Cruz Biotechnology, sc-18043 | 1:200 |
| Anti-HSD3B | Polyclonal | Rabbit | Abcam, ab80363 | 1:10,000 |
| Anti-FGF2 | Polyclonal | Rabbit | Sigma, F3393 | 1:200 |
| Anti-FGFR1 | Polyclonal | Rabbit | Sigma, F5421 | 1:400 |
| Anti-FGFR2 | Polyclonal | Rabbit | Sigma, F6796 | 1:1,000 |
| Anti-VEGFA | Polyclonal | Rabbit | Santa Cruz Biotechnology, sc-152 | 1:200 |
| Anti-VEGFR1 | Monoclonal | Mouse | Abcam, ab9540 | 1:100 |
| Anti-VEGFR2 | Polyclonal | Rabbit | Abcam, ab39256 | 1:400 |

[1]Sigma Chemical Co. (St. Louis, MO); Abcam (Cambridge, UK); Santa Cruz Biotechnology (Santa Cruz, CA).

blot bands for FGF2, VEGFA, and their receptors, STAR, P450scc, HSD3B, and GAPDH are shown in Supplemental Figures S1, S2, and S3 (https://doi.org/10.3168/jds.2019-16644).

### Progesterone Determination

The $P_4$ concentrations in blood plasma were measured in duplicate via direct RIA (RIA Progesterone kit; IM1188, Immunotech, Prague, Czech Republic). The standard curve ranged from 0.1 to 100 ng/mL, and the effective dose for 50% inhibition ($ED_{50}$) was 0.05 ng/mL. The intra- and interassay coefficients of variation were 6.5 and 8.6%, respectively.

### Statistical Analysis

The statistical analyses of the results of mRNA and protein expression were performed using a nonparametric one-way ANOVA Kruskal-Wallis test followed by Dunnett's multiple comparison test (GraphPad Prism ver. 7.0; Graph Pad Software, San Diego, CA). The differences in $P_4$ concentrations in the blood plasma between control groups and experimental groups at the specified time points were calculated using 2-way ANOVA followed by the Bonferroni comparison test, in which the treatments and time of sample collection (hours) were fixed effects with all interactions included (GraphPad Prism). Data are shown as standard errors of means (±SEM). The results were considered to be statistically significant if $P < 0.05$.

## RESULTS

### Changes in mRNA and Protein Expression in Response to Local or Systemic Administration of aPGF$_{2\alpha}$ in Early- and Middle-Stage CL

Figure 1 shows the results for quantitative analysis of mRNA expression of *FGF2*, *FGFR1*, and *FGFR2*. Local and systemic aPGF$_{2\alpha}$ injections upregulated *FGF2* expression in early- and middle-stage CL at 4 h after both treatment routes ($P < 0.05$; Figure 1a). Additionally, expression of *FGF2* was higher after both aPGF$_{2\alpha}$ treatments in early-stage CL compared with that in middle-stage CL ($P < 0.01$; Figure 1a). The *FGFR1* and *FGFR2* expression levels were elevated after local aPGF$_{2\alpha}$ injection into the early-stage CL ($P < 0.01$; Figure 1b, c). Moreover, we observed higher upregulation of *FGFR1* and *FGFR2* expression in early-stage CL in response to local aPGF$_{2\alpha}$ administration compared with that after systemic injection ($P < 0.01$; Figure 1b, c). However, both routes of aPGF$_{2\alpha}$ treatment increased *FGFR2* expression in middle-stage CL ($P < 0.01$; Figure 1c). Additionally, we observed higher *FGFR1* expression after local aPGF$_{2\alpha}$ administration in

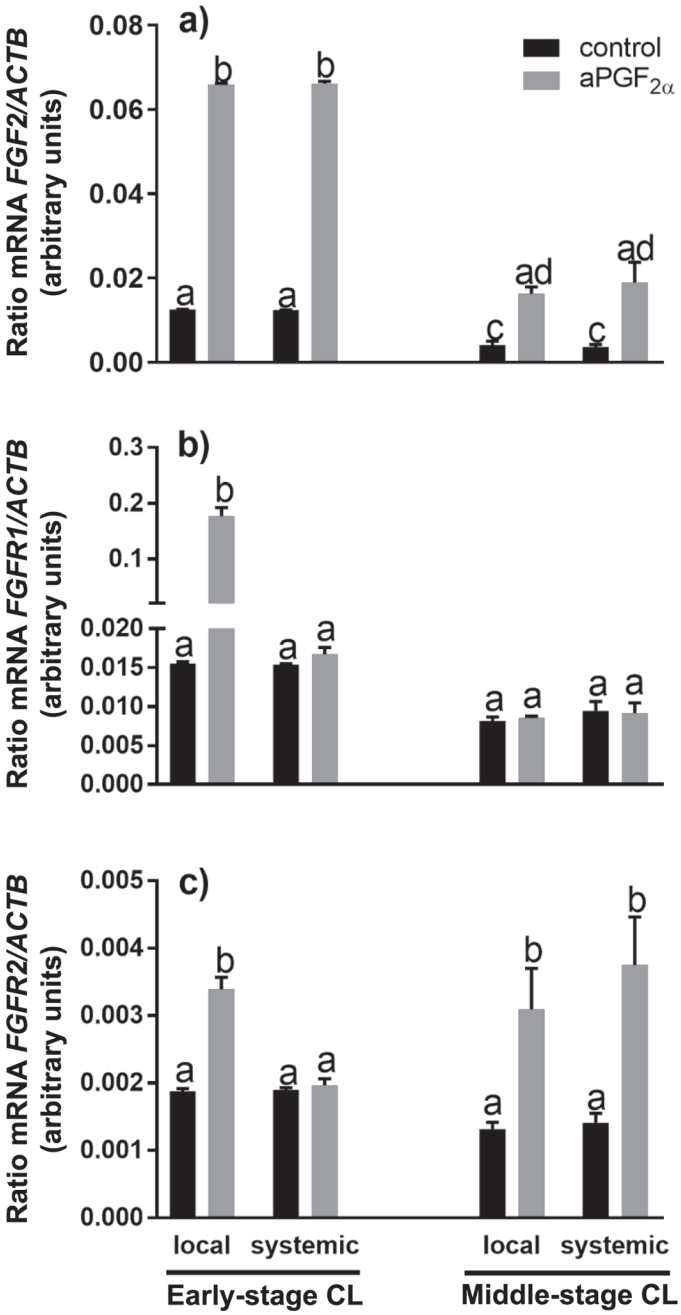


**Figure 1.** Effect of local or systemic $PGF_{2\alpha}$ analog (aPGF$_{2\alpha}$) administration on the mRNA expression of (a) fibroblast growth factor (*FGF2*), (b) fibroblast growth factor receptor 1 (*FGFR1*), and (c) fibroblast growth factor receptor 2 (*FGFR2*) in early- and middle-stage corpora lutea (CL), respectively. The black bars represent the control group, and the gray bars represent intra-CL or i.m. aPGF$_{2\alpha}$ administered groups. Data are the mean ± SEM for 6 samples/treatment. Letters (a–d) indicate statistical differences between treatment groups ($P < 0.05$).

early-stage CL compared with that after local $aPGF_{2\alpha}$ treatment in middle-stage CL ($P < 0.01$; Figure 1b).

Figure 2 shows results of protein expression analysis of FGF2, FGFR1, and FGFR2. Expression of FGF2 increased 4 h after both local and systemic $aPGF_{2\alpha}$ injection in early- and middle-stage CL ($P < 0.001$; Figure 2a). However, FGFR1 expression was upregulated after local $aPGF_{2\alpha}$ injection in both CL stages ($P < 0.05$; Figure 2 b), whereas systemic $aPGF_{2\alpha}$ injection affected FGFR1 expression in middle-stage CL ($P < 0.05$; Figure 2b). Neither route of $aPGF_{2\alpha}$ administration altered FGFR2 expression in early- and middle-stage CL ($P > 0.05$; Figure 2c). Additionally, we observed higher FGF2 expression in early-stage CL after local $aPGF_{2\alpha}$ treatment compared with that in middle-stage CL ($P < 0.01$; Figure 2a).

Figure 3 shows results for the quantitative analysis of mRNA expression of *VEGFA*, *VEGFR1*, and *VEGFR2*. Both local and systemic $aPGF_{2\alpha}$ treatments decreased *VEGFA* expression in early- and middle-stages CL ($P < 0.001$; Figure 3a). We observed higher *VEGFA* expression in early-stage CL after both $aPGF_{2\alpha}$ treatment routes compared with that after local and systemic $aPGF_{2\alpha}$ administration in middle-stage CL ($P < 0.01$; Figure 3a). Additionally, systemic administration of $aPGF_{2\alpha}$ reduced *VEGFA* expression compared with the effect of local $aPGF_{2\alpha}$ treatment in early-stage CL ($P < 0.01$; Figure 3a). Moreover, *VEGFR1* expression was downregulated in both early- and middle-stage CL after both $aPGF_{2\alpha}$ injection routes ($P < 0.05$; Figure 3b); however, its mRNA expression was lower in the early stage CL in response to local $aPGF_{2\alpha}$ injection compared with the systemic treatment route ($P < 0.05$; Figure 3b). Additionally, we observed lower *VEGFR1* expression in early-stage CL after both $aPGF_{2\alpha}$ treatments compared with that after local and systemic $aPGF_{2\alpha}$ administration in middle-stage CL ($P < 0.01$; Figure 3b). In contrast, local $aPGF_{2\alpha}$ injection increased *VEGFR2* expression in early-stage CL ($P < 0.01$; Figure 3c), whereas *VEGFR2* expression was downregulated in middle-stage CL by both $aPGF_{2\alpha}$ treatments ($P < 0.05$; Figure 3c). The expression of *VEGFR2* was lower after systemic $aPGF_{2\alpha}$ administration compared with that after local treatment in both CL stages ($P < 0.01$; Figure 3c). Additionally, we observed lower *VEGFR2* expression in early-stage CL after both $aPGF_{2\alpha}$ treatment routes compared with that after local and systemic $aPGF_{2\alpha}$ administration in middle-stage CL ($P < 0.01$; Figure 3b).

Figure 4 shows the results of protein expression analysis of VEGFA, VEGFR1, and VEGFR2. Both $aPGF_{2\alpha}$ injection routes decreased VEGFA expression in early- and middle-stage CL ($P < 0.05$; Figure 4a),

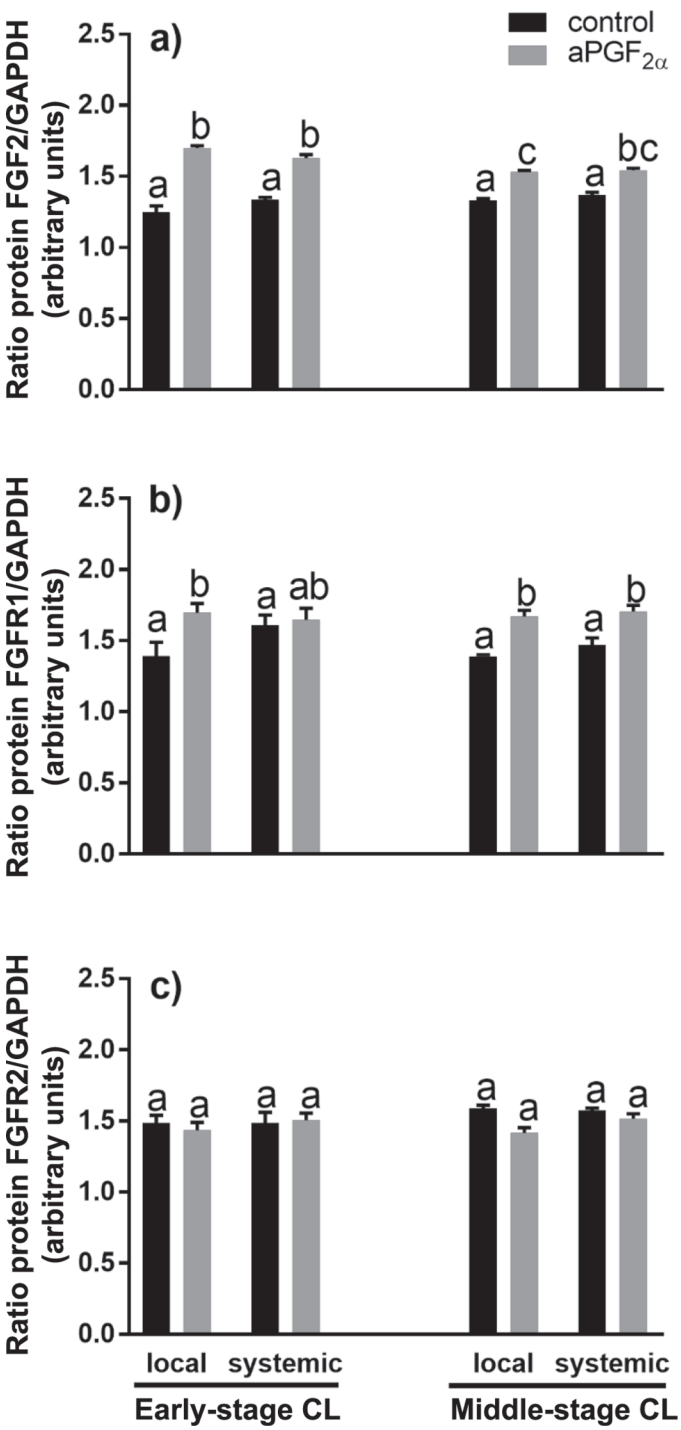


**Figure 2.** Effect of local or systemic $PGF_{2\alpha}$ analog ($aPGF_{2\alpha}$) administration on the protein expression of (a) fibroblast growth factor (FGF2), (b) fibroblast growth factor receptor 1 (FGFR1), and (c) fibroblast growth factor receptor 2 (FGFR2) in early- and middle-stage corpora lutea (CL), respectively. The black bars represent the control group, and the gray bars represent intra-CL or i.m. $aPGF_{2\alpha}$ administered groups. Data are the mean ± SEM for 6 samples/treatment. Letters (a–c) indicate statistical differences between treatment groups ($P < 0.05$).

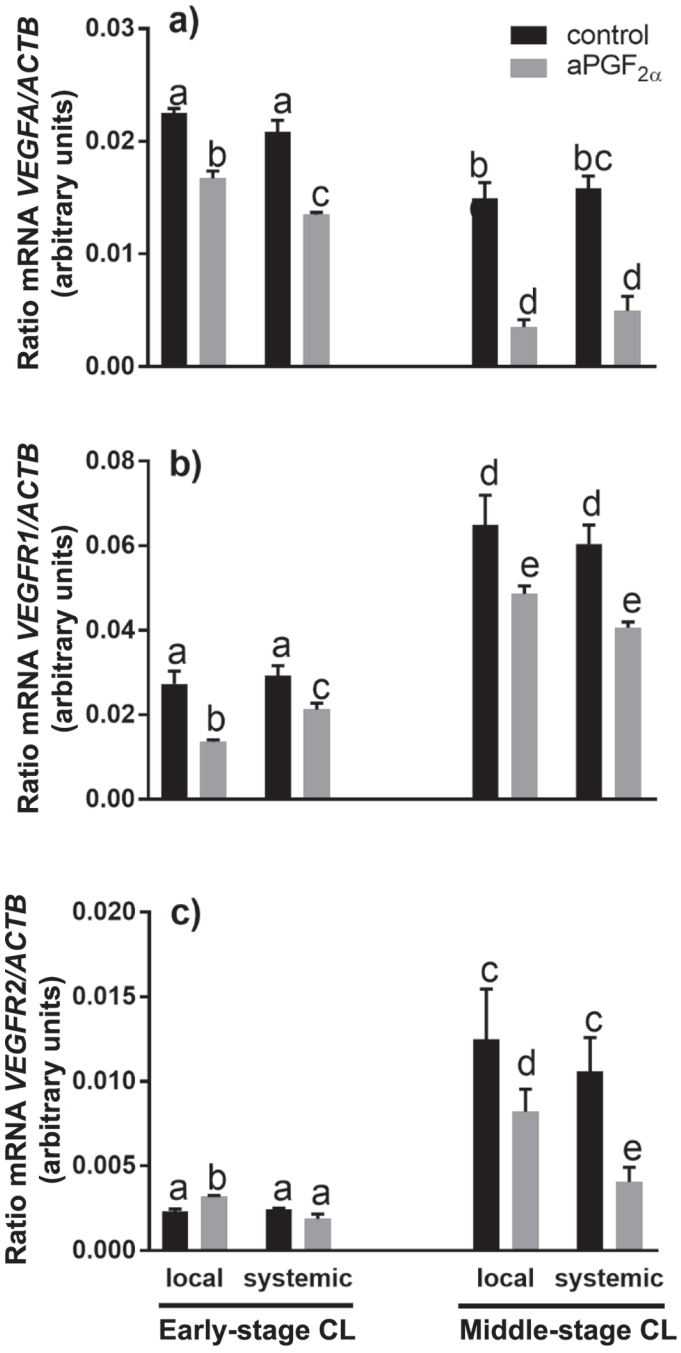


**Figure 3.** Effect of local or systemic $PGF_{2\alpha}$ analog ($aPGF_{2\alpha}$) administration on the mRNA expression of (a) vascular endothelial growth factor A (*VEGFA*), (b) vascular endothelial growth factor receptor 1 (*VEGFR1*) and (c) vascular endothelial growth factor receptor 2 (*VEGFR2*) in early- and middle-stage corpora lutea (CL), respectively. The black bars represent the control group, and the gray bars represent intra-CL or i.m. $aPGF_{2\alpha}$ administered groups. Data are the mean ± SEM for 6 samples/treatment. Letters (a–e) indicate statistical differences between treatment groups ($P < 0.05$).

and systemic $aPGF_{2\alpha}$ administration downregulated VEGFA expression in middle-stage CL compared with local $aPGF_{2\alpha}$ treatment ($P < 0.05$; Figure 4a). Additionally, we observed lower VEGFA expression in middle-stage CL after systemic $aPGF_{2\alpha}$ administration compared with that after systemic $aPGF_{2\alpha}$ treatment in early-stage CL ($P < 0.05$; Figure 4a). Both VEGFR1 and VEGFR2 levels were downregulated after local $aPGF_{2\alpha}$ injection in early-stage CL ($P < 0.05$; Figure 4b, c) and middle-stage CL ($P < 0.01$; Figure 4b, c). Moreover, significantly lower VEGFR1 and VEGFR2 levels were observed after local versus systemic $aPGF_{2\alpha}$ administration in early and middle-stage CL ($P < 0.05$; Figure 4b, c).

### *Changes in STAR, P450scc, and HSD3B mRNA and Protein Expression in Response to Local or Systemic Administration of $aPGF_{2\alpha}$ in Early- and Middle-Stage CL*

Figure 5 shows results for quantitative analysis of mRNA expression of *STAR*, *P450scc*, and *HSD3B*. Both $aPGF_{2\alpha}$ treatment routes increased *STAR* expression in early-stage CL ($P < 0.05$; Figure 5a). However, expression of *STAR* was higher after local $aPGF_{2\alpha}$ injection compared with systemic $aPGF_{2\alpha}$ administration ($P < 0.05$; Figure 5a). Either local or systemic $aPGF_{2\alpha}$ decreased *STAR* expression in middle-stage CL ($P < 0.01$; Figure 5a); however, this effect was greater when $aPGF_{2\alpha}$ was injected systemically ($P < 0.01$; Figure 5a). Additionally, we observed lower *STAR* expression in early-stage CL after both $aPGF_{2\alpha}$ treatment routes compared with that after local and systemic $aPGF_{2\alpha}$ administration in middle-stage CL ($P < 0.01$; Figure 5a).

Neither local nor systemic $aPGF_{2\alpha}$ injection changed *P450scc* mRNA expression in early- and middle-stage CL ($P > 0.05$; Figure 5b).

Local $aPGF_{2\alpha}$ injection upregulated *HSD3B* mRNA expression in early-stage CL ($P < 0.05$; Figure 5c). In contrast, systemic administration of $aPGF_{2\alpha}$ reduced *HSD3B* expression in early- and middle-stage CL ($P < 0.05$; Figure 5c). Therefore, opposite effects of local versus systemic $aPGF_{2\alpha}$ actions on *HSD3B* expression were observed in both early- and middle-stage CL ($P < 0.001$, respectively; Figure 5c).

Figure 6 shows the results of protein expression analysis of STAR, P450scc, and HSD3B. Neither local nor systemic $aPGF_{2\alpha}$ affected STAR, P450scc, or HSD3B expression in early-stage CL ($P > 0.05$; Figure 6a, b, c). However, local $aPGF_{2\alpha}$ injection decreased STAR, P450scc, and HSD3B expression in middle-stage CL ($P < 0.05$; Figure 6a, b, c), whereas systemic $aPGF_{2\alpha}$

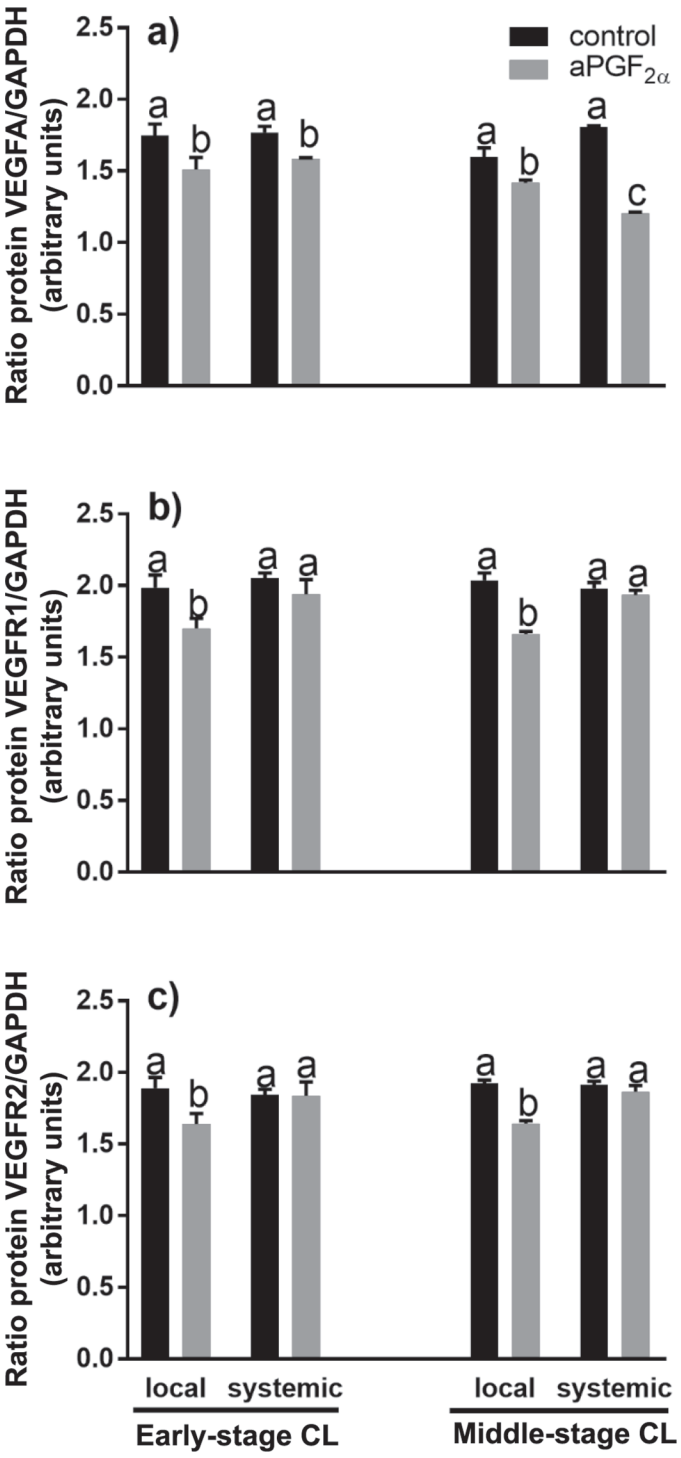


**Figure 4.** Effect of local or systemic $PGF_{2\alpha}$ analog ($aPGF_{2\alpha}$) administration on the protein expression of (a) vascular endothelial growth factor A (VEGFA), (b) vascular endothelial growth factor receptor 1 (VEGFR1) and (c) vascular endothelial growth factor receptor 2 (VEGFR2) in early- and middle-stage corpora lutea (CL), respectively. The black bars represent the control group, and the gray bars represent intra-CL or i.m. $aPGF_{2\alpha}$ administered groups. Data are the mean ± SEM for 6 samples/treatment. Letters (a–c) indicate statistical differences between treatment groups ($P < 0.05$).

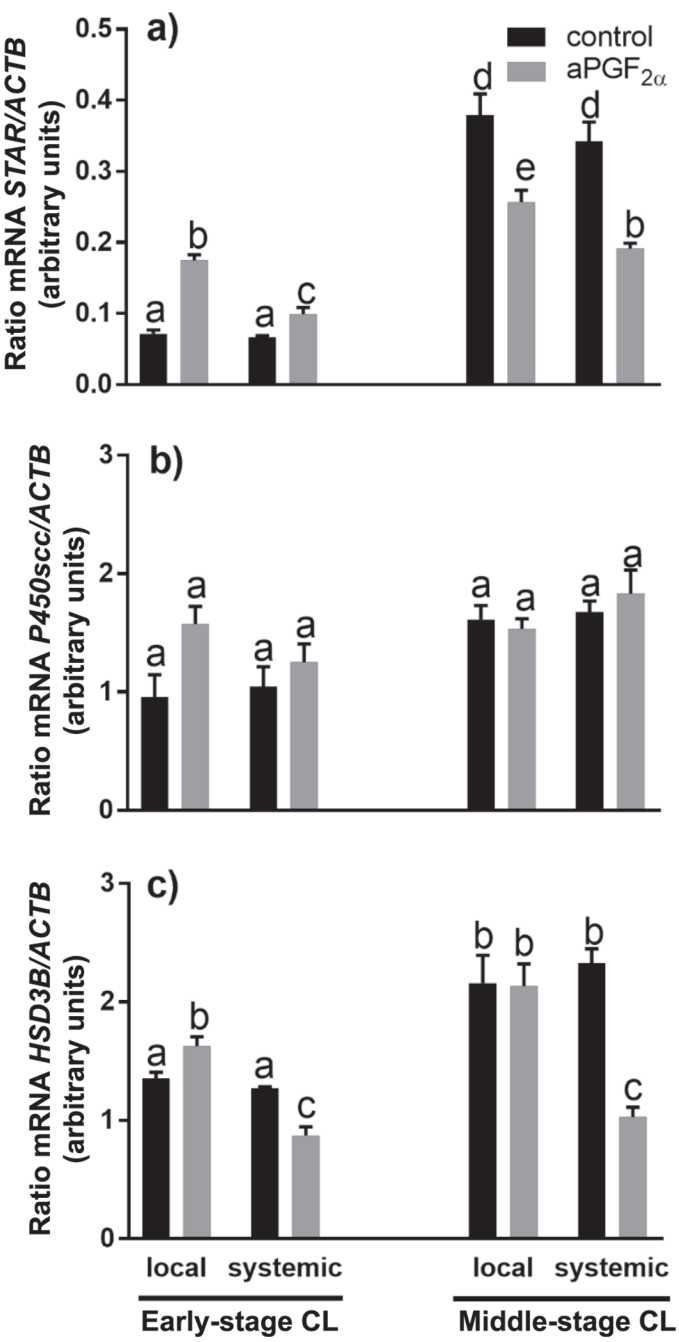


**Figure 5.** Effect of local or systemic $PGF_{2\alpha}$ analog ($aPGF_{2\alpha}$) administration on the mRNA expression of (a) steroidogenic acute regulatory protein (*STAR*), (b) cytochrome P450 family 11 subfamily A member 1 (*P450scc*) and (c) hydroxy-delta-5-steroid dehydrogenase, 3 β- and steroid delta-isomerase 1 (*HSD3B*) in early- and middle-stage corpora lutea (CL), respectively. The black bars represent the control group, and the gray bars represent intra-CL or i.m. $aPGF_{2\alpha}$ administered groups. Data are the mean ± SEM for 6 samples/treatment. Letters (a–e) indicate statistical differences between treatment groups ($P < 0.05$).

treatment downregulated only HSD3B expression in middle-stage CL ($P < 0.001$; Figure 6c). Comparison of local and systemic administration of $aPGF_{2\alpha}$ showed differences in P450scc and HSD3B expression levels in middle-stage CL ($P < 0.05$ and $P < 0.001$; respectively, Figure 6b, c). Moreover, we observed lower STAR and P450scc expression after local $aPGF_{2\alpha}$ injection in middle-stage CL compare with that after local $aPGF_{2\alpha}$ administration in the early stage CL ($P < 0.01$; Figure 6a, b). Additionally, we observed higher HSD3B expression in early-stage CL after both local ($P < 0.05$; Figure 6c) and systemic $aPGF_{2\alpha}$ treatment ($P < 0.01$; Figure 6c) compared with that after both $aPGF_{2\alpha}$ administration routes in middle-stage CL.

### *Changes in Plasma Progesterone Concentration in Response to Local or Systemic Administration of $aPGF_{2\alpha}$ in Early- and Middle-Stage CL*

Figure 7 shows the effect of local or systemic $aPGF_{2\alpha}$ injections on $P_4$ concentrations in blood plasma at the early and middle stages of the estrous cycle. In the early stage, local $aPGF_{2\alpha}$ injection increased the $P_4$ concentration in blood plasma at 0.5 h after treatment ($P < 0.001$; Figure 7a), whereas systemic $aPGF_{2\alpha}$ injection had no effect on $P_4$ concentration ($P > 0.05$; Figure 7c). Following local administration of $aPGF_{2\alpha}$ in the middle stage, circulating $P_4$ levels declined between 1 and 4 h after injection ($P < 0.001$; Figure 7b). However, systemic $aPGF_{2\alpha}$ injection caused a transient increase in plasma $P_4$ concentration at 0.5 h after treatment followed by a decrease at 4 h after treatment ($P < 0.05$; Figure 7d).

## DISCUSSION

The luteolytic role of $aPGF_{2\alpha}$ in cows has already been well described (Hansel and Blair, 1996; Miyamoto et al., 2010). The mechanism underlying stage-specific responses of angiogenesis- and steroidogenesis-modulating genes toward $aPGF_{2\alpha}$ has been the subject of numerous studies (Goravanahally et al., 2009; Shirasuna et al., 2010; Mondal et al., 2011; Atli et al., 2012; Zalman et al., 2012) and is attributed to crosstalk between luteal and nonluteal cells in the bovine CL (Townson et al., 2002; Korzekwa et al., 2006). Importantly, communication between these cells is required for development of the bovine CL as well as its regression (Del Vecchio et al., 1995; Pate, 1995).

Previous studies have examined the effect of $aPGF_{2\alpha}$ treatment on bovine CL at specific time points after its administration (Berisha et al., 2010; Hojo et al., 2016). We elected to collect CL 4 h after $aPGF_{2\alpha}$ treatment

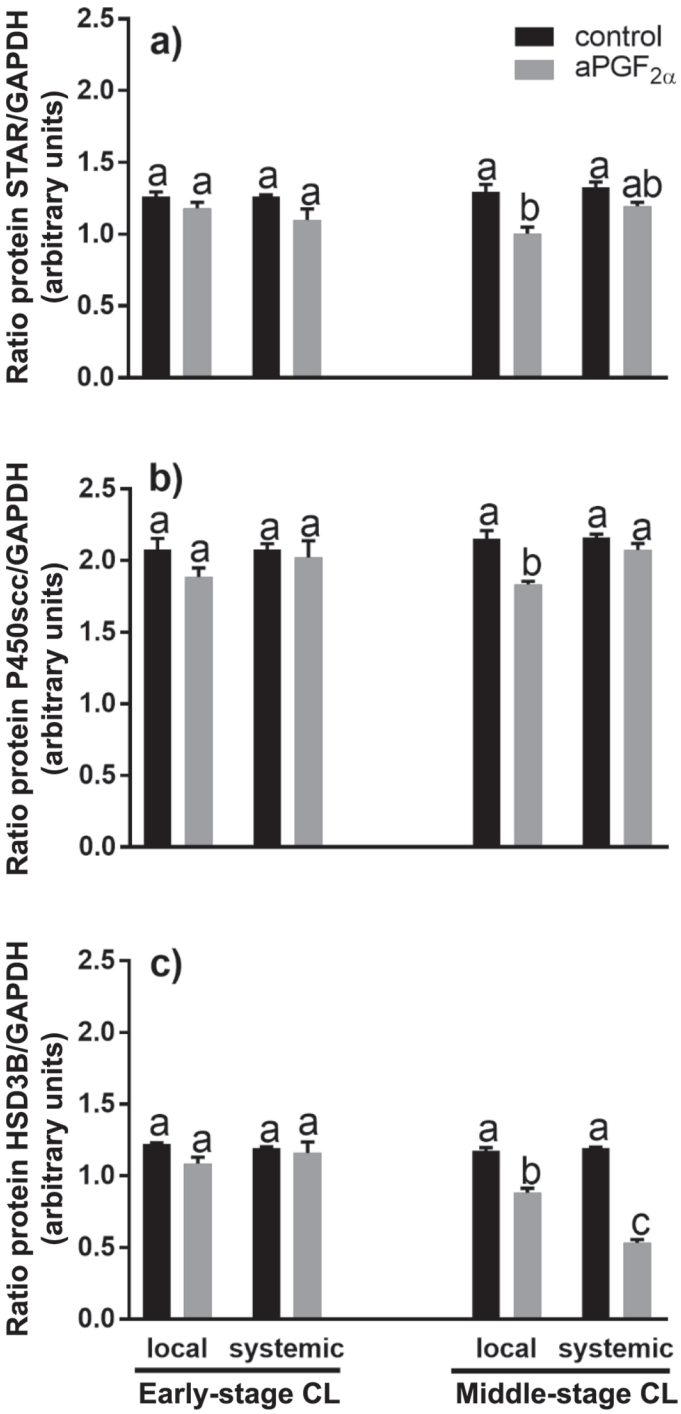


**Figure 6.** Effect of local or systemic $PGF_{2\alpha}$ analog ($aPGF_{2\alpha}$) administration on the protein expression of (a) steroidogenic acute regulatory protein (STAR), (b) cytochrome P450 family 11 subfamily A member 1 (P450scc), and (c) hydroxy-delta-5-steroid dehydrogenase, 3 β- and steroid delta-isomerase 1 (HSD3B) in early- and middle-stage corpora lutea (CL), respectively. The black bars represent the control group, and the gray bars represent intra-CL or i.m. $aPGF_{2\alpha}$ administered groups. Data are the mean ± SEM for 6 samples/treatment. Letters (a–c) indicate statistical differences between treatment groups ($P < 0.05$).

based on previous reports that identified this as a suitable time (Mondal et al., 2011; Zalman et al., 2012; Hojo et al., 2016). Until now, however, there have been no reports indicating a clear difference in the actions of $aPGF_{2\alpha}$ on the CL with regard to local versus systemic administration. Therefore, the results of the present study provide the first evidence in the cow that intra-CL (local) or i.m. (systemic) administration of $aPGF_{2\alpha}$ affects $P_4$ secretion and differentially modulates CL expression of angiogenic and steroidogenic genes during the early and middle stages of the luteal phase.

A previous study reported that mRNA expression of genes related to angiogenesis was upregulated in early-stage CL and downregulated in middle-stage CL in response to systemic $aPGF_{2\alpha}$ treatment in cows (Shirasuna et al., 2010). Our results are in agreement with another report (Zalman et al., 2012) showing that expression levels of 2 main proangiogenic factors, *FGF2*

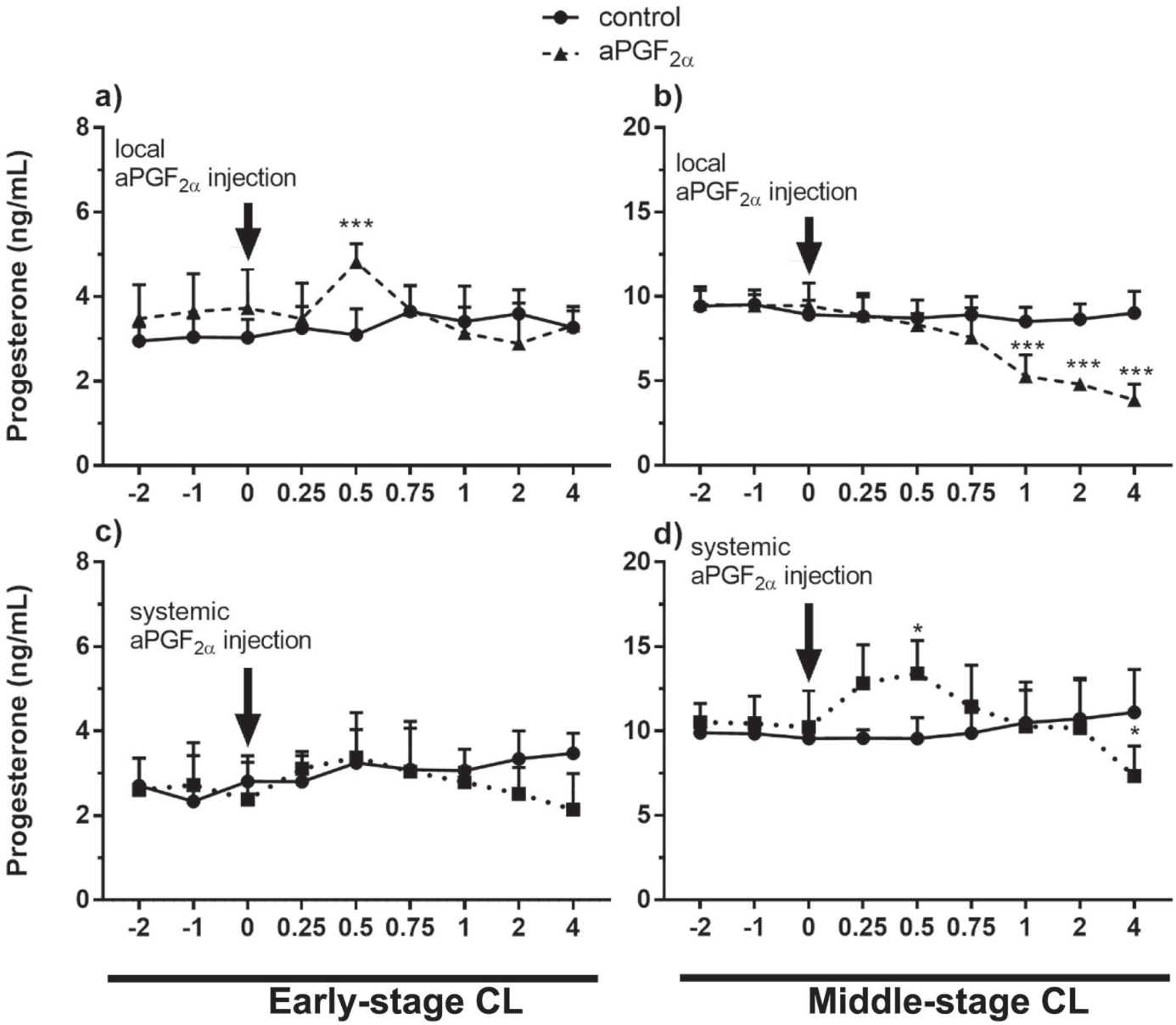


**Figure 7.** Effect of local or systemic $PGF_{2\alpha}$ analog ($aPGF_{2\alpha}$) administration on progesterone ($P_4$) concentrations in blood plasma in cows during the early (a, c) and middle (b, d) stages of the estrous cycle. Data are the mean ± SEM for 6 samples/treatment. Asterisks indicate significant differences between $aPGF_{2\alpha}$-treated groups and control groups at the same time point: $^*P < 0.05$; $^{***}P < 0.001$. CL = corpus luteum.

and *VEGFA*, in bovine middle-stage CL were affected in opposite directions by $aPGF_{2\alpha}$; namely, *FGF2* expression increased and *VEGFA* expression decreased.

During CL formation and growth in cattle, $aPGF_{2\alpha}$ is involved in regulating development of the luteal capillary network (Schams and Berisha, 2004). In contrast, the effect of $aPGF_{2\alpha}$ on angiogenesis in bovine CL may be modulated by FGF2 (Skarzynski et al., 2013). We determined that *FGF2* mRNA expression markedly increased while *VEGFA* mRNA expression decreased in early- and middle-stage CL after both local and systemic $aPGF_{2\alpha}$ treatments. Therefore, the elevation in FGF2 expression due to administration of $aPGF_{2\alpha}$ in the early luteal stage acts as a survival signal for both endothelial (Woad et al., 2009; Shirasuna et al., 2010; Zalman et al., 2012) and steroidogenic cells of the bovine CL (Grazul-Bilska et al., 1995). In the middle of the luteal phase, FGF2 supports CL steroidogenic capacity (Miyamoto et al., 1992; Zalman et al., 2012). Moreover, local neutralization of FGF2 or VEGFA alters genes involved in the regulation of angiogenesis and $P_4$ production, and affects the secretory function of CL during its formation in the cow (Yamashita et al., 2008). Additionally, it was previously reported that FGF2 is immunolocalized to capillary endothelial cells and smooth muscle cells of arteries during the early luteal stage, but expressed exclusively in the cytoplasm of luteal steroidogenic cells during the middle stage (Schams et al., 1994; Neuvians et al., 2004a). Moreover, Schams et al. (1994) suggested that the staining pattern for FGF2 is then characteristic for the late luteal phase, after regression and during pregnancy in bovine CL. In agreement with these findings, we demonstrated elevated *FGF2* expression profiles in early-stage CL compared with middle-stage CL, suggesting that FGF2 expression in the early stage CL is more influenced by $aPGF_{2\alpha}$. Therefore, FGF2 appears to be an important autocrine/paracrine regulator of bovine CL function (Miyamoto et al., 1992; Zalman et al., 2012).

In our study, we found a decline in *VEGFA* mRNA expression after $aPGF_{2\alpha}$-induced luteolysis, which is consistent with previous reports (Berisha et al., 2000; Neuvians et al., 2004a,b). Interestingly, systemic $aPGF_{2\alpha}$ treatment compared with its local administration resulted in a drastic reduction of *VEGFA* mRNA expression in early-stage CL, and VEGFA protein expression in the middle stage CL. Downregulation of *VEGFA* expression after both $aPGF_{2\alpha}$ treatment routes may indicate an ability of $aPGF_{2\alpha}$ to inhibit angiogenesis during CL growth. Moreover, $PGF_{2\alpha}$ downregulated *VEGFA* expression, suggesting inhibition of the angiogenic factor in middle-stage bovine CL (Shirasuna et al., 2010). Therefore, reduced support by angiogenic factors, due to the observed lower mRNA and protein expression of VEGFA levels in middle-stage CL, could destabilize the luteal vasculature, with subsequent reduction of $P_4$ secretion from the CL. These findings suggest that a decrease in one of the main survival factors for endothelial cells (VEGFA) may play a role during functional luteolysis after $aPGF_{2\alpha}$ treatment and possibly that a lack of support of VEGFA results in regression of blood vessels, as suggested by Hanahan (1997).

In vitro treatment with an FGFR1 signaling inhibitor almost totally inhibited the luteal endothelial cell genesis network, confirming that FGF2 is essential for bovine luteal endothelial system formation (Woad et al., 2009, 2012). In agreement with Neuvians et al. (2004a), FGFR1 protein expression in the bovine CL was higher during the early luteal stage in our study. Interestingly, our results showed that local but not systemic administration of $aPGF_{2\alpha}$ increased mRNA expression of *FGFR1*, *FGFR2*, and *VEGFR2* in early-stage CL, whereas expression of *VEGFR1* was downregulated. However, in middle-stage CL, both $aPGF_{2\alpha}$ treatments upregulated *FGFR2* expression and downregulated *VEGFR2* expression. Our results are in agreement with Neuvians et al. (2004a) showing that VEGFR2-expression decreased during luteolysis, resulting in reduced VEGFA functions. We confirmed that expression of FGF2, VEGFA, and their receptors were modulated by both $aPGF_{2\alpha}$ administration routes in early and middle-stage bovine CL.

Previous studies have clearly indicated that key proteins in $P_4$ biosynthesis include STAR, P450scc, and HSD3B (Stocco, 1997). The STAR protein is responsible for the transport of cholesterol to the inner mitochondrial membrane (Stocco, 1997), P450scc converts cholesterol into pregnenolone, and HSD3B converts pregnenolone into $P_4$ (Niswender, 2002). As shown in this study, changes in the mRNA and protein abundance of genes involved in steroidogenesis were accompanied by decreased $P_4$ concentrations in blood plasma during the middle stage, which is in agreement with the report by Tian et al. (1994). In accordance with previous studies by Acosta et al. (2009), we have shown that systemic $aPGF_{2\alpha}$ treatment in the bovine middle stage causes a transient increase in $P_4$ concentrations in blood plasma at 0.5 h, followed by a significant decrease at 4 h. However, Acosta et al. (2002) and Rovani et al. (2017) observed an earlier decrease of $P_4$, which started at 1 or 2 h after injection. The inconsistent results between studies may be attributable to the different concentrations or types of $aPGF_{2\alpha}$ that were used for treatment (cloprostenol vs. dinoprost), meaning that cloprostenol seems to induce earlier luteolysis

than dinoprost. Therefore, the administration method and various types of $aPGF_{2\alpha}$ used may differentially modulate the basal secretion of $P_4$ by the bovine CL (Skarzynski et al., 2009). Shirasuna et al. (2010) reported a reduction in *STAR* mRNA expression after $aPGF_{2\alpha}$ administration in bovine middle-stage CL, whereas there were no changes in its expression during the initial stages of luteolysis (Tian et al., 1994). Our results are in agreement with previous reports of Tsai and Wiltbank (1998) showing that systemic administration of $aPGF_{2\alpha}$ decreases mRNA expression of the final $P_4$-converting enzyme, *HSD3B*, as well as that of *STAR* in bovine middle-stage CL. Moreover, we have shown that protein expression of HSD3B markedly decreases in parallel with the decrease in its mRNA in middle-stage CL after systemic $aPGF_{2\alpha}$ treatment. Notably, expression of *HSD3B* and *STAR* was higher following local $aPGF_{2\alpha}$ treatment compared with that after systemic $aPGF_{2\alpha}$ administration in middle-stage CL. Moreover, we observed that local $aPGF_{2\alpha}$ treatment decreased STAR, P450scc, and HSD3B expression compared with systemic administration. Additionally, local $PGF_{2\alpha}$ application led to a quicker reduction in $P_4$ secretion 1 h after $aPGF_{2\alpha}$ injection compared with the systemic effect of $PGF_{2\alpha}$ in middle-stage CL. These changes in $P_4$ profiles were not detected in early-stage CL, in which luteolysis did not occur.

We have shown that $P_4$ concentration transiently increased 0.5 h after local $aPGF_{2\alpha}$ treatment accompanied by greater *STAR* and *HSD3B* mRNA expression during the early luteal phase. In contrast, we observed a persistently high $P_4$ level in blood plasma during the early luteal phase despite a significant decrease in *HSD3B* mRNA expression after systemic $aPGF_{2\alpha}$ treatment. Therefore, a decrease in *HSD3B* mRNA expression is unlikely to be the cause of decreased luteal steroidogenesis, as suggested by Tsai and Wiltbank (1998).

## CONCLUSIONS

The effects of $aPGF_{2\alpha}$ treatment on the bovine CL depend on stage-specific actions. We have demonstrated for the first time that both local and systemic administration of $aPGF_{2\alpha}$ affect $P_4$ secretion and differentially modulate gene expression of angiogenic and steroidogenic factors in early- and middle-stage CL. At the time of CL formation and development, $aPGF_{2\alpha}$ acting locally may play a luteotrophic role by regulating angiogenesis-related factors. Local $aPGF_{2\alpha}$ treatment increases mRNA expression of *STAR* and *HSD3B* in early-stage CL. Moreover, local $aPGF_{2\alpha}$ treatment enhances mRNA expression of receptors such as *FGFR1*, *FGFR2*, and *VEGFR2* and may be considered a major contributor to the angiogenic response in early-stage bovine CL. However, luteolysis is induced by i.m. $aPGF_{2\alpha}$ injection when the CL is mature (endocrine effect of $aPGF_{2\alpha}$ action), and we demonstrated that local $aPGF_{2\alpha}$ treatment (paracrine effect) may also influence luteolysis. However, the systemic effect of $aPGF_{2\alpha}$ on the mRNA expression of genes participating in steroidogenesis seems to be more substantial than its local effect in middle-stage CL.

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