

## Molecular regulation of progesterone synthesis in the bovine *corpus luteum*

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**ABSTRACT:** In bovine luteal cells, progesterone can directly affect its own synthesis by increasing the activity of  $3\beta$ -HSD. The effect of progesterone on its own secretion coincides with increased expression of the genes for  $3\beta$ -HSD, StAR, and cytochrome P450<sub>scc</sub>. Therefore, progesterone regulates its own synthesis by affecting the activity of the enzymes that take part in luteal steroidogenesis, and also by affecting the expression of the genes for these enzymes. The aims of this study were: (a) to determine whether progesterone affects the expression of the gene for its own receptor, thereby affecting its own synthesis; and (b) to determine whether oxytocin and noradrenaline affect the expression of the genes for the oxytocin receptor (OT-R), the progesterone receptor (P<sub>4</sub>-R), and the  $\beta_2$  receptor ( $\beta_2$ -R), thereby regulating luteal steroidogenesis. Two populations of luteal cells were used in the present study: from 6<sup>th</sup>–10<sup>th</sup> and 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle, which were isolated from *corpus luteum* (CL) from slaughtered cows. The luteal cells were treated for six hours with one of the following hormones: luteinizing hormone (LH; 100 ng/ml); progesterone (P<sub>4</sub>; 10<sup>-5</sup>M); progesterone antagonist (aP<sub>4</sub>; 10<sup>-5</sup>M); noradrenaline (NA; 10<sup>-5</sup>M); or actinomycin D (ActD; 500 ng/ml). After treatment, the medium was collected for the determination of progesterone concentration. With LH, the P<sub>4</sub> concentration in the medium increased with both 6<sup>th</sup>–10<sup>th</sup> and 11<sup>th</sup>–16<sup>th</sup> days. None of the other treatments affected the progesterone concentration of the medium. The level of expression of the genes for OT-R, P<sub>4</sub>-R and  $\beta_2$ -R were determined. Total RNA was extracted from cells, treated with DNase, and subjected to reverse transcription. Treatment with luteinizing hormone was the only treatment that increased the level of expression of the gene for P<sub>4</sub>-R in both 6<sup>th</sup>–10<sup>th</sup> and 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle. Both treatment with luteinizing hormone and treatment with progesterone increased the level of expression of the gene for OT-R in 6<sup>th</sup>–10<sup>th</sup> days. The basal level of expression of the gene for OT-R was higher in 6<sup>th</sup>–10<sup>th</sup> days than in 11<sup>th</sup>–16<sup>th</sup> days. This suggests that there is positive feedback between progesterone and oxytocin, with both playing a role as a local, intra-ovarian factor that enhances the function of the *corpus luteum*.

**Keywords:** steroidogenesis; *corpus luteum*; oxytocin receptor; progesterone receptor;  $\beta_2$  receptor; cattle

The *corpus luteum* is an endocrine gland that forms from follicular cells after ovulation. It regulates the duration of the estrous cycle and maintains gestation in many species, including cows (Baird, 1992; Davis and Rueda, 2002). The main function of the *corpus luteum* is the secretion of progesterone, which is synthesized from cholesterol. Cholesterol can be derived from the diet or be synthesized *de*

*novo* from acetyl CoA (Bloch, 1965; Shroepfer, 1982). Cholesterol is transported to the ovaries by lipoproteins (HDL and LDL). It is taken into the cells by endocytosis. Cholesterol esters are hydrolyzed to free cholesterol (Niswender et al., 2000). The cholesterol molecules are transported across the double mitochondrial membrane by Steroidogenic Acute Regulatory protein (StAR). In the mitochon-

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drion, the cholesterol is converted to pregnenolone by cytochrome P450 side chain cleavage (P450scc). In the endoplasmic reticulum, the pregnenolone is finally converted to progesterone by 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD).

At a concentration of 10<sup>-5</sup>M, progesterone affects its own synthesis by stimulating the activity of cytochrome P450scc and 3 $\beta$ -HSD. However, this happens only from the sixth to tenth days of the estrous cycle (Rothchild, 1996; Kotwica et al., 1998, 2004). Progesterone also affects its own synthesis by inducing the genes for StAR, cytochrome P450scc and 3 $\beta$ -HSD from the 6<sup>th</sup>–10<sup>th</sup> and 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle (Rekawiecki et al., 2005).

When noradrenaline is administered, the excretion of progesterone and oxytocin from the *corpus luteum* increases within a few minutes, both *in vivo* and *in vitro* (Kotwica and Skarzynski, 1993; Bogacki and Kotwica, 1999; Miszkiel and Kotwica, 2001). Furthermore, noradrenaline that is synthesized in the *corpus luteum* from dopamine can stimulate the activity of 3 $\beta$ -HSD and peptidyl glycine-amidating mono-oxygenase (PGA) (Bogacki and Kotwica, 1999; Kotwica and Bogacki, 1999). PGA is a key enzyme in oxytocin synthesis. On the other hand, noradrenaline has no effect on the expression of the genes for StAR, cytochrome P450scc, or 3 $\beta$ -HSD (Rekawiecki et al., 2005).

The aims of this study were the following: to determine whether progesterone affects the expression of the gene for its own receptor, thereby affecting its own synthesis; and to determine whether oxytocin and noradrenaline affect the expression of the genes for the oxytocin receptor (OT-R), the progesterone receptor (P<sub>4</sub>-R), and the  $\beta_2$  receptor ( $\beta_2$ -R), thereby regulating luteal steroidogenesis.

## MATERIAL AND METHODS

### Harvest of corpora lutea and preparation of luteal cells

Unless otherwise stated, all chemicals were obtained from Sigma (Poznan, Poland). All media were enriched with gentamycin (20  $\mu$ g/ml; ICN, Rzeszow, Poland). Corpora lutea from non-gravid cows were harvested at a commercial slaughterhouse and transported to the laboratory in ice-cold PBS within one hour of death. Luteal cells were obtained by perfusing the corpora lutea with mixture

of collagenase IA (1 mg/ml) and DNase I (5  $\mu$ g/ml) as described by Okuda et al. (1992).

Two populations of luteal cells isolated from *corpus luteum* were used in the present study: 6<sup>th</sup>–10<sup>th</sup> and 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle. The stage of the estrous cycle was estimated on the basis of the criteria described by Ireland et al. (1980). Cell viability was estimated using 0.04% trypan blue dye. Only cells with viability above 80% were used in this study.

Luteal cells (106/ml) were suspended in 4 ml of DMEM/Ham's-F12 medium supplemented with 10% FCS. The cells were placed in six-well microtiter plates (Corning Inc., Schiphol-Rijk, Netherlands). The cells were pre-incubated for 24 h to allow the cells to attach themselves to the bottoms of the wells. The medium was discarded, and the cells were washed twice with M-199 containing 0.1% BSA. The wells were then filled with 4 ml of DMEM/Ham's-F12 medium supplemented with 0.1% BSA, 20  $\mu$ g/ml ascorbic acids, 5  $\mu$ g/ml transferrin, and 5 ng/ml sodium selenite (ICN, Rzeszow, Poland). The cells were cultured at 38°C under an atmosphere of air containing 5% CO<sub>2</sub> (Heraeus BB-6060, Hanau, Germany). The relative humidity was kept at 100%. For each experiment, luteal cells were pooled from two or three corpora lutea. Each treatment was applied in four replications.

### Stimulation of the cells

Luteal cells were stimulated for 6 h with LH (100 ng/ml), P<sub>4</sub> (10<sup>-5</sup>M), aP<sub>4</sub> (10<sup>-5</sup>M), NA (10<sup>-5</sup>M) and Actinomycin D (ActD) (500 ng/ml). Cells incubated without hormones served as the control. After treatment, the medium was collected for the determination of progesterone concentration. The cells were stored at -80°C pending RNA isolation.

### Progesterone assay

Progesterone concentration in the medium was determined by EIA as described by Prakash et al. (1987). The assay was carried out using a reader plate (Multiscan EX, Labsystem, Helsinki, Finland). Absorbance was measured at 450 nm. Progesterone labeled with horse radish peroxidase (HRP) was used at final dilution of 1:80 000. Progesterone antiserum was used at a final dilution of 1:50 000 (Kotwica et al., 1994). The range of the standard

curve was 0.1 to 25 ng/ml, and the sensitivity of the procedure was 0.15 ng/ml. The mean intra-assay and mean inter-assay coefficients of variation for the control samples were 6.1% and 8.4 %, respectively. There was a strong positive correlation between the added and measured amounts of hormone ( $n = 7$ ;  $r = 0.96$ ).

### RNA isolation and semi-quantitative RT-PCR assays

Total RNA was extracted from cells in accordance with the procedure described by Chomczynski and Sacchi (1987). The isolated RNA was stored at  $-80^{\circ}\text{C}$  pending further analysis. The purity and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm. 1  $\mu\text{g}$  of RNA was treated with DNase and subjected to reverse transcription for 60 min at  $42^{\circ}\text{C}$  in 20  $\mu\text{l}$  of reaction mixture. The reaction mixture contained the following: RT-buffer (50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM  $\text{MgCl}_2$  and 5mM dithiothreitol; Fermentas, Vilnius, Lithuania); 10mM of each dNTP; 500 ng of anchored oligo (dT)23 primers; and 200 U of reverse transcriptase (Fermentas, Vilnius, Lithuania). The reaction was terminated by heating for 10 min at  $70^{\circ}\text{C}$ .

Based on the gene sequences in the GenBank homepage, primers were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA). The primers were synthesized by TIB MolBiol (Poznan, Poland). The sequences of the primers, the expected PCR product length and the number of cycles are shown in Table 1.

The reaction mixture contained the following: 2  $\mu\text{l}$  cDNA derived from one sample from the reverse

transcription step described above; 12.5  $\mu\text{l}$  REDTaq ReadyMix PCR Reaction Mix (1.5 units Taq DNA polymerase, 10mM Tris-HCl, 50mM KCl, 1.5mM  $\text{MgCl}_2$ , 0.001% gelatin, 0.2mM dNTP) and 1 $\mu\text{M}$  of both PCR primers for each of the genes studied. The optimal conditions for PCR were determined in a series of preliminary studies, which included a determination of the linearity of reaction. PCR was carried out as follows: denaturation for 1 min at  $94^{\circ}\text{C}$ ; annealing for 1 min at  $55^{\circ}\text{C}$ ; primers attachment and extension for 1 min at  $72^{\circ}\text{C}$ ; and final extension for 10 min  $72^{\circ}\text{C}$ ).

The PCR reaction products were separated by electrophoresis on 15% polyacrylamide gel together with well-characterized molecular mass standards (Marker M1, DNA, Gdansk, Poland). The intensity of each band was quantified using Kodak EDAS 290 software. Relative amounts of the PCR products were estimated by normalizing the signal intensities of the gene products in relation to the housekeeping gene 18s RNA product.

### Data analysis

For each of the genes studied, the mean and standard error of the progesterone concentration and the amount of mRNA were calculated. All data were statistically elaborated using one-way ANOVA, followed by means separation using the Newman-Keuls test at  $P < 0.5$ . All calculations were carried out using the (GraphPad Prism 2.0) software package (GraphPad Software, Inc., San Diego, CA, USA). Basal levels of gene expression in corpora lutea from days 6 to 10 and from days 11 to 16 of the estrous cycle were also compared using the Newman-Keuls test.

Table 1. Forward and reverse primer sequences, expected amplicon length and number of cycles used in PCR. Every primer set is designed according the accession number in the The Entrez Nucleotide database

Primer	Sequence (5'–3')	Amplicon length (bp)	Number of cycles	Accession number
OT-R	forward: CGCGCCTCTTCTTCTTCATG reverse: CCGTAGAAGCGGAACGTGAT	106	26	NM_174134
PR-R	forward: GCATGTCGCCTTAGAAAGTGC reverse: TTCGGCCTCCAAGAACCAT	52	30	AJ557823
$\beta_2$ -R	forward: CGTCTACTCCAGGGTGTTCCT reverse: CCTGCTCCACTTGACTGACGT	101	31	NM_174231
18s RNA	forward: GTTCGATTCCGGAGAGGGA reverse: CCTTCCTTGATGTGGTAGCC	51	21	AF176811

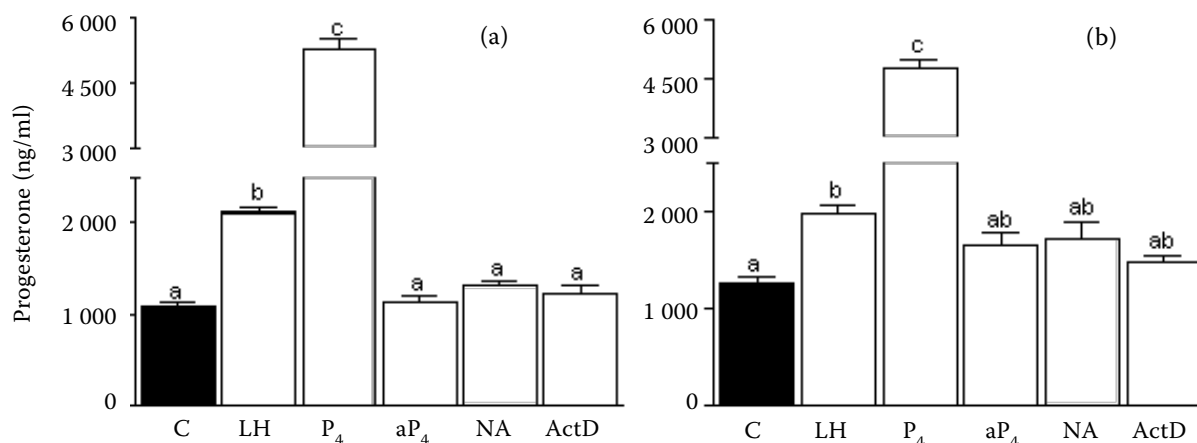


Figure 1. Progesterone concentration in Early Cycle (a) and Mid Cycle (b) luteal cells after treatment with luteinizing hormone (LH), progesterone (P<sub>4</sub>), progesterone antagonist (aP<sub>4</sub>), noradrenaline (NA), or actinomycin D (ActD) in comparison to untreated cells (C). Bars marked with different superscripts are different at  $P < 0.05$

## RESULTS

After treatment with luteinizing hormone, the secretion of progesterone increased in both 6<sup>th</sup> to 10<sup>th</sup> ( $P < 0.05$ ) and 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle ( $P < 0.001$ ). None of the other treatments affected progesterone concentration as compared to the control (Figure 1). In 6<sup>th</sup>–10<sup>th</sup> days, the level of OT-R mRNA was elevated after treatment with

luteinizing hormone ( $P < 0.01$ ) and progesterone ( $P < 0.01$ ). In 11<sup>th</sup>–16<sup>th</sup> days, none of the treatments affected the level of OT-R mRNA. (Figure 2). The level of P<sub>4</sub>-R mRNA was elevated after treatment with luteinizing hormone in both 6<sup>th</sup>–10<sup>th</sup> ( $P < 0.05$ ) and 11<sup>th</sup>–16<sup>th</sup> ( $P < 0.05$ ) days of the estrous cycle (Figure 3). The level of  $\beta_2$ -R mRNA decreased after treatment with actinomycin D in 6<sup>th</sup>–10<sup>th</sup> days cells ( $P < 0.01$ ). None of the other treatments affected

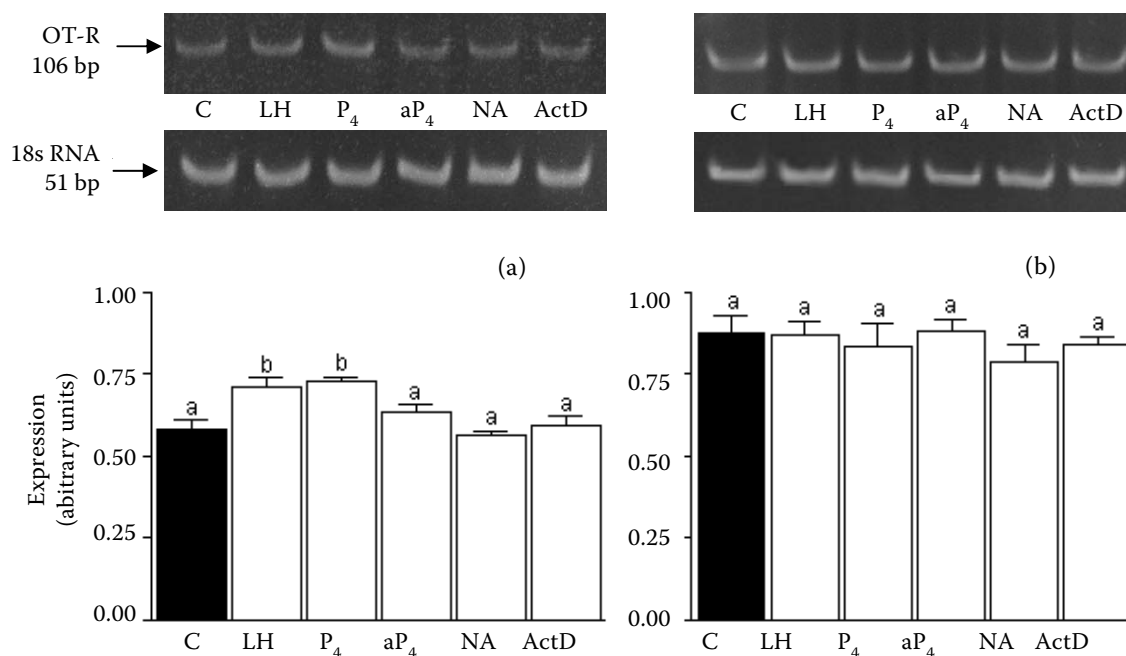


Figure 2. OT-R mRNA level in Early Cycle (a) and Mid Cycle (b) luteal cells after treatment with luteinizing hormone (LH), progesterone (P<sub>4</sub>), progesterone antagonist (aP<sub>4</sub>), noradrenaline (NA), or actinomycin D (ActD) in comparison to untreated cells (C). Upper panel: representative image of gel. Lower panel: densitometric analyses of OT-R relative to 18s RNA. Bars marked with different superscripts are different at  $P < 0.05$

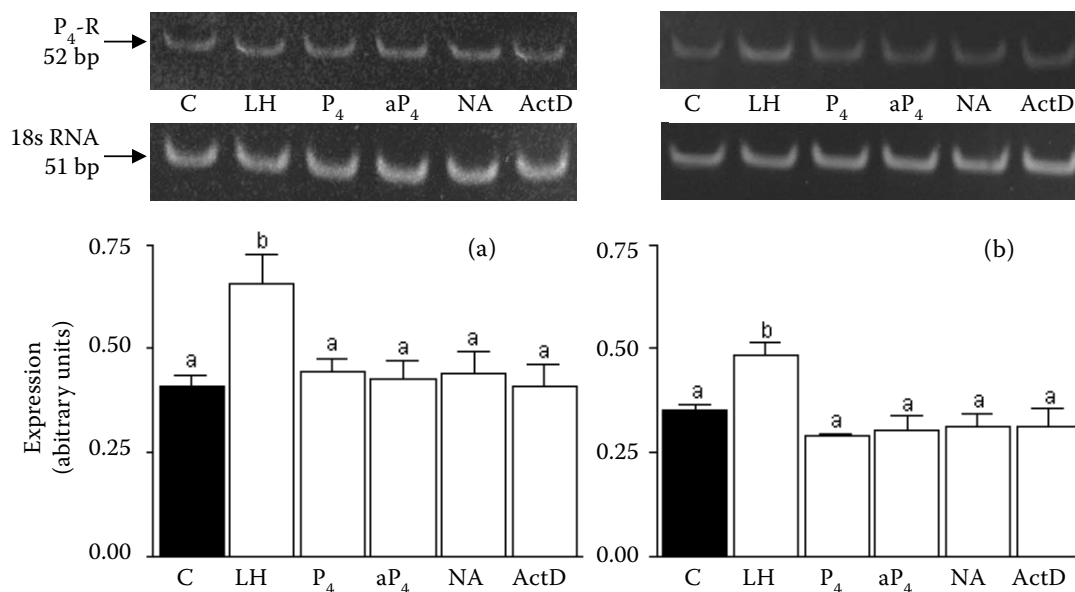


Figure 3.  $P_4$ -R mRNA level in Early Cycle (a) and Mid Cycle (b) luteal cells after treatment with luteinizing hormone (LH), progesterone ( $P_4$ ), progesterone antagonist ( $aP_4$ ), noradrenaline (NA), or actinomycin D (ActD) in comparison to untreated cells (C). Upper panel: representative image of gel. Lower panel: densitometric analyses of  $P_4$ -R relative to 18s RNA. Bars marked with different superscripts are different at  $P < 0.05$

the level of  $\beta_2$ -R mRNA in both stages of luteal cells (Figure 4). Basal expression of the gene for OT-R was lower in 6<sup>th</sup>–10<sup>th</sup> days than in 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle ( $P < 0.001$ ). Basal expression of the

gene for  $P_4$ -R was the same in both studied stages of the estrous cycle. Basal expression of the gene for  $\beta_2$ -R was higher in 6<sup>th</sup>–10<sup>th</sup> days than in 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle ( $P < 0.001$ ) (Table 2).

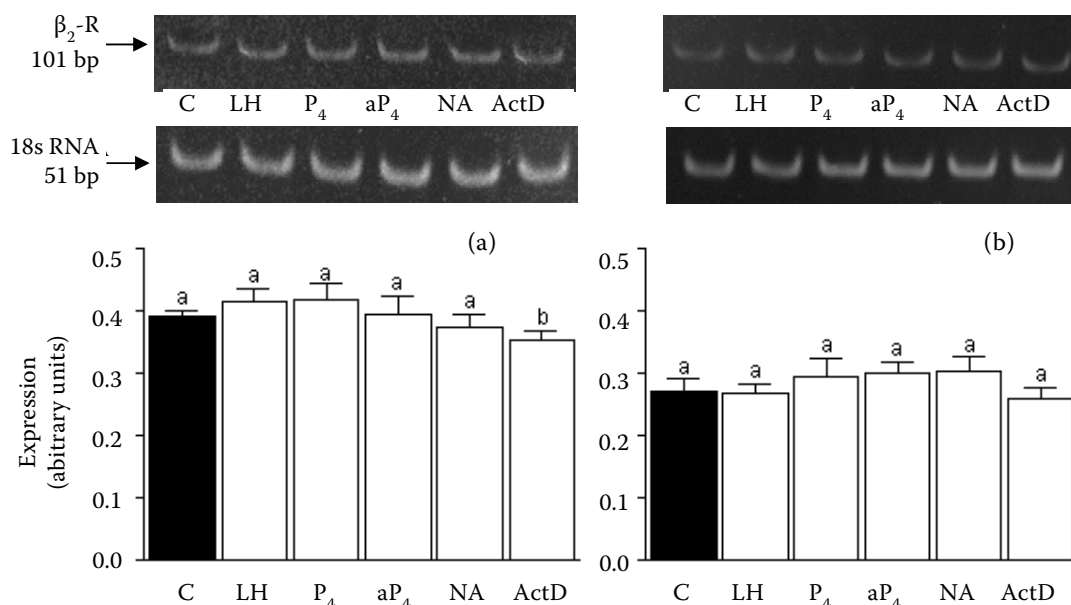


Figure 4.  $\beta_2$ -R mRNA level in Early Cycle (a) and Mid Cycle (b) luteal cells after treatment with luteinizing hormone (LH), progesterone ( $P_4$ ), progesterone antagonist ( $aP_4$ ), noradrenaline (NA), or actinomycin D (ActD) in comparison to untreated cells (C). Upper panel: representative image of gel. Lower panel: densitometric analyses of  $\beta_2$ -R relative to 18s RNA. Bars marked with different superscripts are different at  $P < 0.05$

Table 2. Comparison of the basic expression of OT-R, P<sub>4</sub>-R and  $\beta_2$ -R genes in cultured bovine luteal cells from days 6–10 and 11–16 of the estrous cycle. Results in line with different superscripts are different ( $P < 0.05$ )

Receptor	Days of estrous cycle	
	6–10	11–16
OT-R	0.581 $\pm$ 0.029 <sup>a</sup>	0.874 $\pm$ 0.054 <sup>b</sup>
P <sub>4</sub> -R	0.412 $\pm$ 0.029 <sup>a</sup>	0.354 $\pm$ 0.012 <sup>a</sup>
$\beta_2$ -R	0.389 $\pm$ 0.011 <sup>a</sup>	0.270 $\pm$ 0.019 <sup>b</sup>

## DISCUSSION

The cells to be used in this investigation were selected on the basis of progesterone concentration. Treatment with luteinizing hormone was the only treatment that increased progesterone secretion in 6<sup>th</sup>–10<sup>th</sup> and 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle. Luteinizing hormone also increased the level of expression of the gene for OT-R, but only in 6<sup>th</sup>–10<sup>th</sup> days. Luteinizing hormone is the main luteotropic hormone in cattle (Baird, 1992; Niswender et al., 2000). The pre-ovulatory surge in luteinizing hormone appears to be the main initiator of ovarian oxytocin and progesterone production (Schams, 1987). In cows, oxytocin increases the level of progesterone, especially from the 5<sup>th</sup>–7<sup>th</sup> days of the estrous cycle. The effect decreased from 8<sup>th</sup>–12<sup>th</sup> to 15<sup>th</sup>–18<sup>th</sup> days of the estrous cycle (Miyamoto and Schams, 1991).

In the present study, treatment with luteinizing hormone increased the expression of the gene for OT-R in cells from 6<sup>th</sup>–10<sup>th</sup> days. The increased number of receptors may be another mechanism by which luteinizing protein and oxytocin increase progesterone production.

Treatment with luteinizing hormone also increased the expression of the gene for P<sub>4</sub>-R in both studied stages. This indicates that luteinizing hormone enhances the ability of progesterone to stimulate its own synthesis, as had been previously shown (Kotwica et al., 2004). On the other hand, progesterone alone did not affect expression of the gene for P<sub>4</sub>-R in either 6<sup>th</sup>–10<sup>th</sup> or 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle. Previous results showed that progesterone can directly affect its own synthesis in bovine luteal cells from the 6<sup>th</sup>–10<sup>th</sup> days of the estrous cycle by increasing the activity of 3 $\beta$ -HSD

inactivity (Kotwica et al., 1998, 2004). Furthermore, the effect of progesterone on its own secretion coincides with an increase in the level of expression of the genes for 3 $\beta$ -HSD, StAR, and cytochrome P450scc (Rekawiecki et al., 2005). Therefore, progesterone regulates its own synthesis by affecting the activity of the enzymes that take part in luteal steroidogenesis, and also by affecting the expression of the genes for these enzymes.

None of the treatments affected the expression of the gene for  $\beta_2$ -R. This agrees well with our earlier observations that noradrenaline did not affect either the expression of the genes for StAR, 3 $\beta$ -HSD and cytochrome P450scc, or the levels of these proteins themselves (Rekawiecki et al., 2005). However, the fact that noradrenaline does not have a molecular effect on gene expression does not exclude the possibility that it has a luteotropic influence on the *corpus luteum*. In heifers, noradrenaline increases the secretion of oxytocin and progesterone both *in vivo* and *in vitro* (Kotwica et al., 1991; Kotwica and Skarzynski, 1993; Jaroszewski and Kotwica, 1994; Miszkiewicz and Kotwica, 2001). It also affects progesterone synthesis by acting on  $\beta_2$ -R and by activating cAMP (Lefkowitz and Caron, 1987; Miszkiewicz and Kotwica, 2001). Subsequently, it up-regulates 3 $\beta$ -HSD and cytochrome P450scc (Bogacki and Kotwica, 1999; Miszkiewicz and Kotwica, 2001). Furthermore, noradrenaline increases lipolysis and luteal blood flow (Wiltbank et al., 1990). In this way, it increases the supply of serum-derived lipoprotein to the *corpus luteum*. This increases the pool of cholesterol available for steroidogenesis (Williams, 1989). In this regard, noradrenaline plays an important role of the luteotropic complex which supports the function of the *corpus luteum*.

Actinomycin D may promote apoptosis and decrease viability in bovine granulosa and endometrial cells (Wrobel and Kotwica, 2005; Mlynarczuk and Kotwica, 2006). This is why it was used as a negative control in the present study. Nevertheless, actinomycin D did not have any clear deleterious effects in this study, probably because the treatment time was too short.

Progesterone antagonist also did not have a clear deleterious effect on the treated cells. Treatment for 6 hours of luteal slices with onapristone inhibits 3 $\beta$ -HSD activity in 5<sup>th</sup>–10<sup>th</sup> days of the estrous cycle (Kotwica et al., 2004). However, RU-486 may behave like progesterone when it acts on P<sub>4</sub>-R isophorm B, and behave like an antagonist when it acts on P<sub>4</sub>-R isophorm A (Rothchild, 1996). This

paradoxical effect may have affected the results of the present study.

The level of expression for the gene for OT-R was higher in the second half of the estrous cycle. This agrees well with previous studies on cow in which the binding of oxytocin to the luteal cell membrane was highest from the eighth to the twelfth days of the estrous cycle, coinciding with the peak in ovarian oxytocin secretion (Wathes et al., 1984; Okuda et al., 1992). The results of the present study support the hypothesis that oxytocin plays a role in luteolysis from the eighth to the twelfth days of the estrous cycle.

The level of expression of the gene for  $P_4$ -R was statistically the same in both studied stages of cells. In the earlier studies in which the amount of  $P_4$ -R in bovine luteal cells was measured both by immunocytochemistry and by Western blotting, it was highest in 6<sup>th</sup>–10<sup>th</sup> days, that is, when the ability of progesterone to stimulate 3 $\beta$ -HSD activity was highest (Kotwica et al., 1998, 2004). However, in another study on bovine corpora lutea, there was no significant change in the level of expression for the gene for  $P_4$ -R, either during the estrous cycle or during pregnancy (Berisha et al., 2002). The results of the present study suggest that  $P_4$ -R plays an autocrine/paracrine role in regulating the development and maintenance of the *corpus luteum*.

The basal level of expression of the gene for  $\beta_2$ -R was higher in 6<sup>th</sup>–10<sup>th</sup> than in 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle. This agrees well with an earlier study in which a radio-receptor assay was used (Pesta et al., 1994). In heifers, the administration of noradrenaline stimulates the secretion of progesterone and oxytocin during all stages of the development of the *corpus luteum*. The effect is greatest in from the fifth to the eight days of the estrous cycle (Jaroszewski and Kotwica, 1994). This suggests that  $\beta$ -adrenergic stimulation regulates function of the *corpus luteum* in the early stages of the luteal phase.

In conclusion, treatment with luteinizing hormone was the only treatment that increased the level of expression of the gene for  $P_4$ -R in both studied stages of bovine luteal cells. Both treatment with luteinizing hormone and treatment with progesterone increased the level of expression of the gene for OT-R in 6<sup>th</sup>–10<sup>th</sup> of the estrous cycle. The basal level of expression of the gene for OT-R was higher in 6<sup>th</sup>–10<sup>th</sup> than in 11<sup>th</sup>–16<sup>th</sup> days of the estrous cycle. This suggests that there is positive feedback between progesterone and oxytocin,

with both playing a role as a local, intra-ovarian factor that enhances the function of the *corpus luteum*.

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