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Original article

Effect of cortisol on neurophysin I/oxytocin and peptidyl glycine- α -amidating mono-oxygenase mRNA expression in bovine luteal and granulosa cells

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Abstract

Cortisol stimulates the synthesis and secretion of oxytocin (OT) from bovine granulosa and luteal cells, but the molecular mechanisms of cortisol action remain unknown. In this study, granulosa cells or luteal cells from days 1-5 and 11-15 of the oestrous cycle were incubated for 4 or 8 h with cortisol (1×10^{-5} , 1×10^{-7} M). After testing cell viability and hormone secretion (OT, progesterone, estradiol), we studied the effect of cortisol on mRNA expression for precursor of OT (NP-I/OT) and peptidyl glycine- α -amidating mono-oxygenase (PGA). The influence of RU 486 (1×10^{-5} M), a progesterone receptor blocker and inhibitor of the glucocorticosteroid receptor (GR), on the expression for both genes was tested. Cortisol increased the mRNA expression for NP-I/OT and PGA in granulosa cells and stimulated the expression for NP-I/OT mRNA in luteal cells obtained from days 1-5 and days 11-15 of the oestrous cycle. Expression for PGA mRNA was increased only in luteal cells from days 11-15 of the oestrous cycle. In addition, RU 486 blocked the cortisol-stimulated mRNA expression for NP-I/OT and PGA in both types of cells. These data suggest that cortisol affects OT synthesis and secretion in bovine ovarian cells, by acting on the expression of key genes, that may impair ovary function.

Key words: cattle, ovary, cortisol, oxytocin synthesis

Introduction

Oxytocin (OT) is synthesised and secreted from bovine granulosa cells and luteal cells, and participates in the regulation of the oestrous cycle (Stormshak 2003). As an autocrine factor, OT supports the growth and maturation of the ovarian follicles (Voss and Fortune 1993, Jo and Fortune 2003). In addition,

OT is involved in the ovulation and luteinisation of granulosa and theca cells (Tallam et al. 2000). Oxytocin also stimulates progesterone (P_4) secretion in the early and middle stages of the oestrous cycle (Miyamoto and Schams 1991). At the end of the oestrous cycle, OT is involved in the pulsatile secretion of endometrial $PGF_{2\alpha}$, and thus participates in luteolysis (Flint and Sheldrick 1983, Kotwica et al. 1998, McCracken et al. 1999).

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Besides the prostaglandins and oestrogens in luteal cells and LH, and FSH in granulosa cells (Wathes 1989, Kotwica et al. 1990), cortisol was also found to increase OT secretion from bovine ovarian cells (Luck 1988, Mlynarczuk and Kotwica 2005, 2006). Glucocorticosteroids (GCs) are steroid hormones produced in the zona fasciculata and the reticulate of the adrenal cortex. They are involved in a number of physiological processes, such as stress response, inflammation, immune function, hydroelectrolyte balance, behaviour and reproduction (Nozaki 2001, Schuerholz et al. 2007). Cortisol, which is the main GC in cows, can prolong their oestrous cycle (Kanchev et al. 1976, Stoebel and Moberg 1982), affect ovulation by inhibition of the LH surge (Suter and Schwartz 1985, Macfarlane et al. 2000) and is considered an anti-luteolytic factor in the corpus luteum (CL) (McCracken et al. 1999, Lee et al. 2007, Komiyama et al. 2008). Under physiological conditions, GCs initiate parturition; in contrast, high levels of cortisol, released during stress, can cause abortions in humans and animals (Thong et al. 1993, Short et al. 1995). One potential explanation for this phenomenon is that cortisol may excessively stimulate OT secretion, which can disrupt "progesterone block" in the myometrium. However, the molecular mechanism underlying the effect of cortisol on the synthesis and secretion of OT from bovine ovarian cells is unknown.

Consequently, the aim of these studies was to determine the influence of cortisol on the synthesis and secretion of OT by bovine luteal and granulosa cells. We tested the influence of cortisol and its receptor antagonist RU 486 during 4 or 8 h of cell stimulation to capture the first changes in mRNA expression for neurophysin I/oxytocin (NP-I/OT), the precursor of OT, and peptidyl glycine- α -amidating mono-oxygenase (PGA), which participates in post-translational OT processing. Because OT is involved in CL function in both the early and middle stages of the oestrous cycle, CL from days 1-5 and 11-15 was used. Note that RU 486 is a progesterone receptor blocker (Liu et al. 1987), which can non-specifically block GR (Jacquot et al. 2008).

Materials and Methods

Collection of ovaries

Ovaries from cows or mature heifers at a defined stage of the oestrous cycle, as described by Ireland et al. (1980) and Fields and Fields (1996), were collected from a commercial slaughterhouse within 15-20 min of killing the animals. Ovaries were placed in an ice-cold 0.9% NaCl solution containing penicillin (10

IU/ml), streptomycin (100 μ g/ml), amphotericin (2 μ g/ml), and L-glutamine (100 μ g/ml) and then transported to the laboratory on ice within 1 hour. Luteal cells were obtained from corpora lutea in two stages: days 1-5 and days 11-15 of the oestrous cycle. Granulosa cells were isolated from ovarian follicles with a <1> cm in diameter and without regard to oestrous cycle phase. Atretic follicles were examined and eliminated on the basis of criteria described by Henderson et al. (1987). All materials were purchased from Sigma (Poland), unless otherwise stated.

Preparation of granulosa and luteal cells

Granulosa cells were obtained from 12-14 follicles ($n = 4$ cows) by vigorous aspiration of the follicular fluid. Luteal cells from days 1-5 of the cycle were obtained from the CL ($n = 4$ cows) by dispersion of split luteal tissue with an enzyme mixture (1 mg/ml Collagenase IA and 5 μ g/ml DNase I). Cells from days 11-15 of the oestrous cycle were obtained by perfusion of the ovarian artery with the same enzyme mixture as described by Okuda et al. (1992). Cell suspensions were collected in conical tubes, washed with M-199 containing Earle's salt and 0.1% BSA and centrifuged at 1200x g for 10 min at 4°C. The supernatant was removed, the cells were resuspended in DMEM/F-12 HAM enriched with 10% FCS and then sieved through nylons. The viability of the cells, estimated by exclusion of trypan blue dye (0.04%), was approximately 80%. Cell suspensions were transferred into 6-well (5×10^5 /ml, for mRNA expression studies) and 24-well plates (2.5×10^5 /ml, for hormone secretion studies) and preincubated for 24 h. All plates used were Nunclon Δ -surface (NUNC, Denmark). After preincubation, the cells were washed twice with M-199 containing 0.1% BSA to remove cells that did not attach to the well bottom. The cells were then covered with DMEM/F-12 HAM supplemented with 0.1% BSA, ascorbic acid (20 μ g/ml), transferrin (5 μ g/ml) and sodium selenite (5 ng/ml, ICN), and used for further experiments. Cells were cultured at 38°C in air containing 5% CO₂ and 100% humidity (Memmert, Germany). All media excluded phenol red and were enriched with gentamycin (40 mg/ml) and amphotericin (20 mg/ml; ICN Pharmaceuticals, USA).

Preliminary studies

The influence of cortisol on cell viability was studied using the *TOX-1 In Vitro Toxicology Assay Kit MTT Based* test. Cortisol was applied in doses of 1×10^{-5} M or 1×10^{-7} M (as used by Luck 1988) and in

combination with RU 486 (1×10^{-5} M; Mlynarczuk and Kotwica 2006) for 72 h of incubation. Actinomycin D (500 ng/ml) was used as a negative control. Absorbance of the enzymatic reaction product was determined using a Multiscan EX (Labsystems, Finland) at a wavelength of 570 nm. Neither the dose of cortisol nor cortisol combined with RU 486 affected cell viability (data not shown); therefore, we used these concentrations of hormone in further studies.

Experiment 1: *Effect of cortisol on the secretion of E_2 or P_4 and OT from granulosa or luteal cells*

Granulosa or luteal cells from days 1-5 and 11-15 of the oestrous cycle were incubated with cortisol (1×10^{-5} M or 1×10^{-7} M) for 48 h, as was done in our previous studies (Mlynarczuk et al. 2005, 2010). Gonadotropins, FSH (100 ng/ml; AFP 5679C) or LH (100 ng/ml, AFP-4261A) were used as positive controls for granulosa and luteal cells, respectively. Estradiol or P_4 and OT were measured in the media collected after termination of incubation.

Experiment 2: *Effect of cortisol on the mRNA expression for NP-I/OT and PGA in granulosa and luteal cells*

Granulosa or luteal cells from days 1-5 and 11-15 of the oestrous cycle were incubated with cortisol (1×10^{-5} M or 1×10^{-7} M) for 4 or 8 h to determine mRNA expression for NP-I/OT and PGA as was previously reported by Mlynarczuk et al. (2009, 2010).

Experiment 3: *Effect of RU486 on the cortisol-stimulated mRNA expression for NP-I/OT and PGA in granulosa and luteal cells*

Granulosa or luteal cells from days 1-5 and 11-15 of the oestrous cycle were incubated for 1 h with RU 486 (1×10^{-5} M), as in a previously established protocol (Mlynarczuk and Kotwica, 2006), followed by application of cortisol (1×10^{-5} M or 1×10^{-7} M) to the media. Changes in mRNA expression were observed after the shorter treatment period tested in Exp. 2; therefore, incubation was terminated after 4 h and the cells were used for determining the mRNA expression for NP-I/OT and PGA.

Hormone determination

Hormone concentrations in the culture media were determined using an enzyme immunoassay according to the protocol of Prakash et al. (1987). Progesterone and E_2 were labelled with horseradish peroxidase, and OT was biotin-labelled with a Biotin Labelling Kit (Boehringer Mannheim). The cross-reaction of used antisera against P_4 , OT and E_2 was shown previously (Kotwica and Skarzynski 1993, Kotwica et al. 1994, Mlynarczuk and Kotwica 2005).

The range of the standard curve, intra- and inter-assay coefficients of variation, and the relationship between added and measured amounts ($n=4$), expressed as the coefficient of regression, were as follows: for P_4 , 0.39-100 ng/ml, 8.5%, 9.7% and $r=0.98$; for OT, 3.9-2 000 pg/ml, 8.2%, 10.4% and $r=0.93$; for E_2 , 6.25 – 1600 pg/ml, 9.9%, 11.9% and $r=0.89$. All assays were performed in 96-well plates covered with ovine anti-rabbit γ -globulin, obtained in our department. The absorbance at a wavelength of 450 nm was measured using a Multiscan MX plate reader and the results were initially analysed using a Genesis Lite 3.0 (Labsystems, Finland).

RT-PCR analysis

Total mRNA was isolated from cell cultures using a Total RNA kit (A&A Biotechnology, Poland) according to the procedure of Chomczynski and Sacchi (1987). The concentration and purity of the samples were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA; absorbance $A_{260/280}$) and analysed using the NanoDrop 1000 v.3.7.1 software. All samples had an absorbance ratio between 1.8 and 2.0. Samples of RNA were diluted with autoclaved water to obtain the same concentration of RNA (0.5 μ g/8 μ l of solution) from all wells in the culture. Reverse transcription (RT) was performed in a thermocycler (MJ Mini, BioRad, Poland) at 42°C for 60 min using the Reverse Transcriptase enzyme (Fermentas, Lithuania). The reaction was terminated by heating for 10 min at 70°C. The obtained cDNA was amplified by PCR, which was conducted using 12.5 μ l of Readymix RedTaq PCR Reaction Mix (total volume of sample – 25 μ l). Each tube contained 2 μ l of cDNA, 50 μ M of both primers in 0.5 μ l volume and 9.5 μ l of water. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as an internal standard. G3PDH is constitutively expressed in granulosa-derived and theca-derived luteal cells and has been used effectively in studies on the regulation of gene expression in luteal cells (Rękawiecki and Kotwica 2007). Primers for NP-I/OT (forward: ccgctgtacattcagaac, reverse: gtctcagtgtgggagaagg, size of product: 329 bp), PGA (forward: accagcagtgaccagaatc, reverse: acgatgggtgcttaatctcg, size of product: 314 bp) and G3PDH (forward: tccacacacctgttctgta, reverse: tgttccagtagtccacc, size of product: 318 bp) were the same as those used by Mlynarczuk et al. (2009). Primers were synthesised at the Institute of Biochemistry and Biophysics (PAN, Poland) based on the gene sequences available in GenBank (NCBI) using the primer3 software

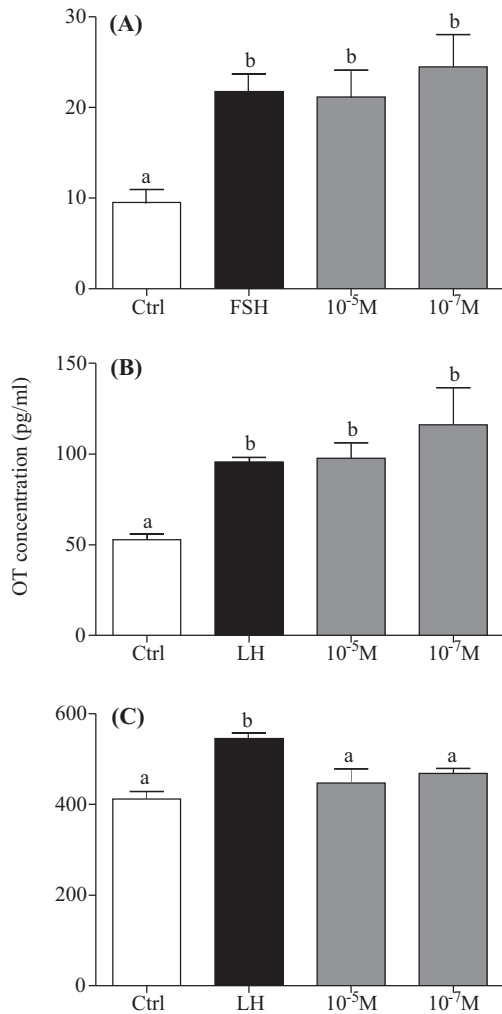


Fig. 1. Influence of cortisol (1×10^{-5} , 1×10^{-7} M) on OT secretion from granulosa ($n = 4$ cows) (A) and luteal cells ($n = 4$ cows) from days 1-5 (B) and 11-15 (C) of the oestrous cycle. FSH (A) and LH (B,C) were used as positive controls. Bars with different superscript letters are significantly different ($P < 0.05$).

(<http://frodo.wi.mit.edu/primer3/input.htm>). The PCR amplification was performed for 25 cycles for NPI/OT and PGA, and 23 cycles for G3PDH. The conditions of one cycle of PCR were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. RT-PCR amplification products and well-characterised molecular mass standards (Marker M1, DNA, Poland) were separated on a 2% agarose gel (Maximus, Germany) by electrophoresis, stained with ethidium bromide and visualised under UV light (DNR, Israel). EDAS 290 software (Kodak, USA) was used to quantify the intensity of bands. Relative expression of the genes was estimated by normalising the signal intensity of each gene product to the G3PDH intensity.

Statistical analysis

The mean (\pm SEM) mRNA expression and hormone concentrations were analysed by one-way ANOVA followed by Newman-Keuls, a post-hoc test for comparison of normally distributed data (Graph-Pad PRISM 4.0 software, USA).

Results

Cortisol (1×10^{-5} and 1×10^{-7} M) significantly increased OT secretion from granulosa and luteal cells on days 1-5 of the oestrous cycle ($P < 0.05$; Fig. 1A,B). Both doses of cortisol also increased ($P < 0.05$) the secretion of P₄ from luteal cells at days 1-5 of the oestrous cycle (Fig. 2B), whereas E₂ secretion ($P < 0.05$) from granulosa cells was stimulated only by the higher dose of cortisol (Fig. 2A).

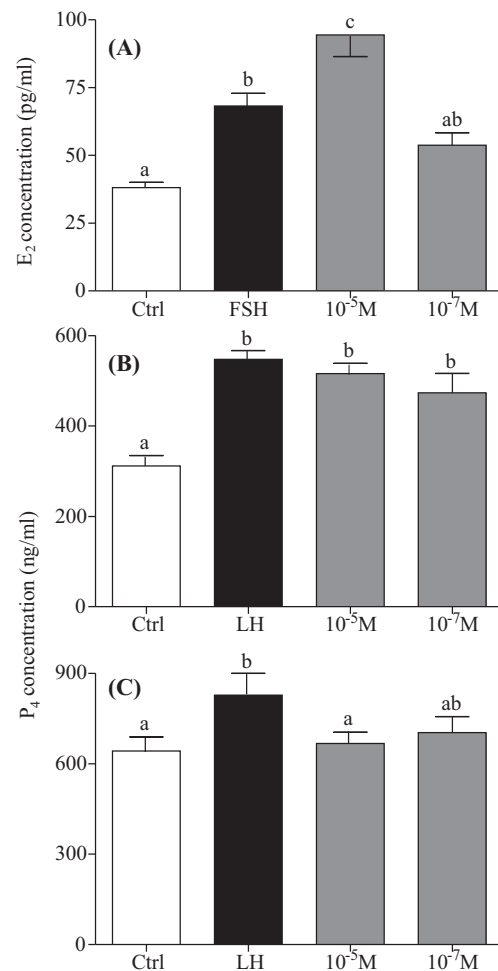


Fig. 2. Influence of cortisol (1×10^{-5} , 1×10^{-7} M) on secretion of E₂ from granulosa ($n = 4$ cows) (A) and P₄ from luteal cells ($n = 4$ cows) from days 1-5 (B) and 11-15 (C) of the oestrous cycle. FSH (A) and LH (B,C) were used as positive controls. Bars with different superscript letters are significantly different ($P < 0.05$).

After 4 and 8 h of incubation, cortisol (1×10^{-5} , 1×10^{-7} M) increased ($P < 0.05$) mRNA expression for the oxytocin precursor NP-I/OT and PGA in granulosa cells (Fig. 3). Moreover, the higher dose of cortisol (1×10^{-5} M) stimulated ($P < 0.05$) mRNA expression for NP-I/OT in luteal cells from days 1-5 (Fig. 4) and 11-15 of the oestrous cycle (Fig. 5) for the same incubation time. In the luteal cells from days 11-15 of the cycle, mRNA expression for PGA increased ($P < 0.05$) after 4 and 8 h of stimulation with cortisol at a dose of 1×10^{-7} M, whereas the 1×10^{-5} M dose was effective only after 8 h of incubation (Fig. 5).

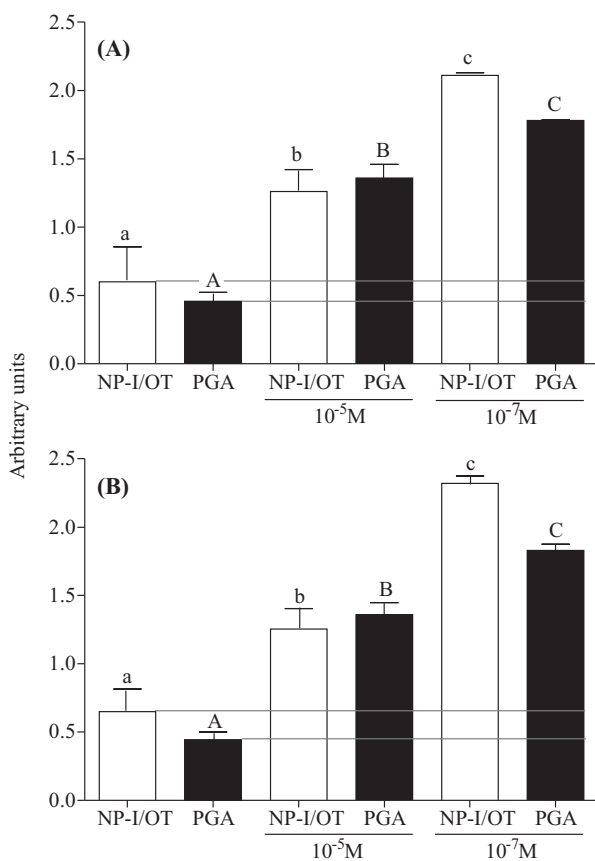


Fig. 3. Influence of cortisol (1×10^{-5} , 1×10^{-7} M) on mRNA expression for NP-I/OT (open bars) and PGA (black bars) in granulosa cells ($n = 4$ cows) after 4 (A) and 8 h (B) of incubation compared to control values (horizontal line). Bars with different superscript letters are significantly different ($P < 0.05$).

RU 486 decreased ($P < 0.05$) mRNA expression for NP-I/OT in granulosa cells stimulated by a 1×10^{-5} M dose of cortisol (Fig. 6A). The effect of both doses of cortisol on mRNA expression for PGA was reduced ($P < 0.05$) by RU 486 (Fig. 6B). Used blocker also decreased ($P < 0.05$) cortisol-stimulated (1×10^{-5} M) mRNA expression for NP-I/OT in luteal cells from days 1-5 of the oestrous cycle (Fig. 7). The mRNA

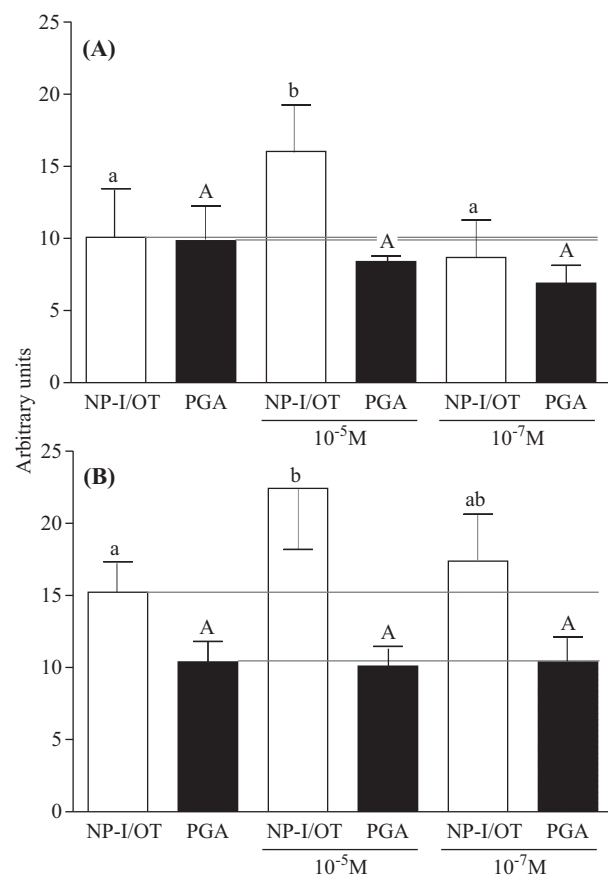


Fig. 4. Influence of cortisol (1×10^{-5} , 1×10^{-7} M) on the mRNA expression for NP-I/OT (open bars) and PGA (black bars) in luteal cells ($n = 4$ cows) from days 1-5 of the oestrous cycle after 4 h (A) and 8 h (B) of incubation compared to control values (horizontal line). Bars with different superscript letters are significantly different ($P < 0.05$).

expression for PGA in cells from this stage was not tested because there was no cortisol effect (Exp. 2). In luteal cells from days 11-15 of the cycle, RU 486 inhibited ($P < 0.05$) the stimulating effect of both doses of cortisol on mRNA expression for NP-I/OT (Fig. 8A) and the effect of the higher dose of cortisol (1×10^{-5} M) on the mRNA expression for PGA (Fig. 8B).

Discussion

Cortisol, either alone or in combination with the GR blocker RU 486, did not affect the viability of granulosa and luteal cells, which is in agreement with earlier observations (Spicer and Chamberlain 1998, Mlynarczuk and Kotwica 2005). Furthermore, changes in hormone concentrations in response to LH or FSH and cortisol indicate that the cells maintain their physiological properties. Therefore, we conclude that the results obtained in this study were not caused by the cytotoxic effect.

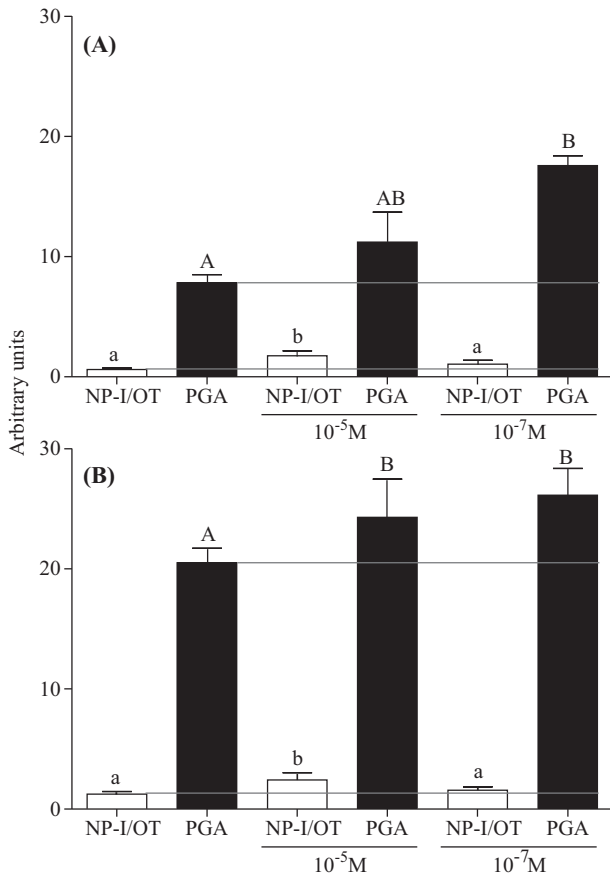


Fig. 5. Influence of cortisol (1×10^{-5} , 1×10^{-7} M) on the mRNA expression for NP-I/OT (open bars) and PGA (black bars) in luteal cells ($n = 4$ cows) from days 11-15 of the oestrous cycle after 4 h (A) and 8 h (B) of incubation compared to control values (horizontal line). Bars with different superscript letters are significantly different ($P < 0.05$).

Our data show that cortisol can influence the synthesis and secretion of OT from granulosa and luteal cells in cows; cortisol may impair many reproductive processes, such as growth, maturation and ovulation of follicles, the establishment of the corpus luteum and luteolysis, as well as bovine fertility (Flint and Sheldrick 1983, Thatcher et al. 1994, Tallam et al. 2000, Bah et al. 2006). Oxytocin also affects steroidogenesis in the ovary; around the time of ovulation, ovarian OT stimulates the synthesis of P_4 and decreases androstenedione (A_4) and E_2 production, which causes the luteinisation of theca and granulosa cells (Miyamoto and Schams 1991, Lioutas et al. 1997). In addition, OT may affect adrenal steroidogenesis and change the secretion of glucocorticoids, depending on the phase of the oestrous cycle (Kotwica et al. 2004).

When incubated with cortisol, the mRNA expression for NP-I/OT and PGA was increased in granulosa cells and luteal cells from days 11-15 of the oestrous cycle, which coincided with increased OT

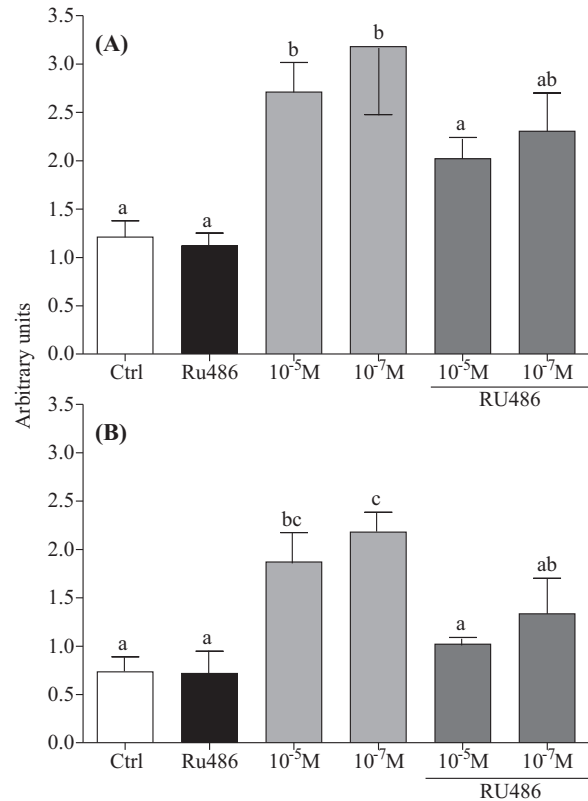


Fig. 6. Influence of RU 486 (1×10^{-5} M) on the mRNA expression for NP-I/OT (A) and PGA (B) in granulosa cells ($n = 4$ cows) stimulated by cortisol (1×10^{-5} , 1×10^{-7} M) after 4 h of incubation. Bars with different superscript letters are significantly different ($P < 0.05$).

secretion in the same cells. Moreover, the effect of cortisol stimulation on mRNA expression in luteal cells was intensified during the 8 h incubation time. Differences in the levels of NP-I/OT mRNA between the two luteal stages of the cycle correspond to physiological processes occurring in the luteal cells. The highest concentration of OT, along with the synthesis and storage of OT prohormone in granules of the Golgi apparatus, occurs at the beginning of the cycle, i.e., 2-3 days after ovulation, and then declines in days 6-7 of the oestrous cycle (Flint and Sheldrick 1983, Jones and Flint 1988). Furthermore, activity of PGA gradually increases to a maximum between days 8-16 after ovulation (Sheldrick and Flint 1989); this explains why PGA mRNA expression is unchanged in cells from the early luteal stage, but it is at high levels in cells from days 11-15 of the oestrous cycle. Hence, GCs can extend the time of OT precursor synthesis and increase OT secretion by acting on the genomic pathway.

Similar to our studies, glucocorticosteroids were found to stimulate P_4 and E_2 secretion from luteal and granulosa cells, respectively (Adashi et al. 1981, Spicer and Chamberlain 1998). Moreover, low con-

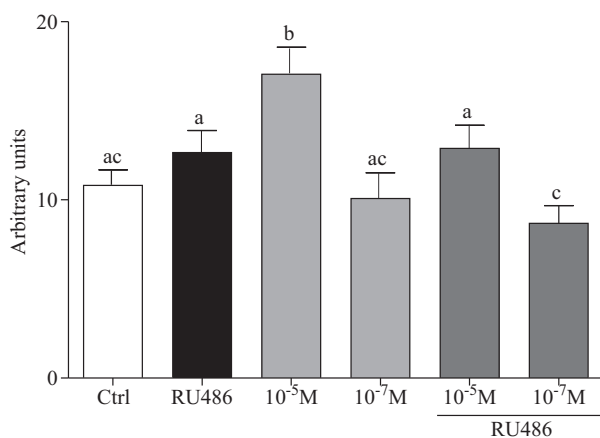


Fig. 7. Influence of RU 486 (1×10^{-5} M) on the cortisol-stimulated (1×10^{-5} , 1×10^{-7} M) mRNA expression for NP-I/OT in luteal cells ($n = 4$ cows) from days 1-5 of the oestrous cycle after 4 h of incubation. Bars with different superscript letters are significantly different ($P < 0.05$).

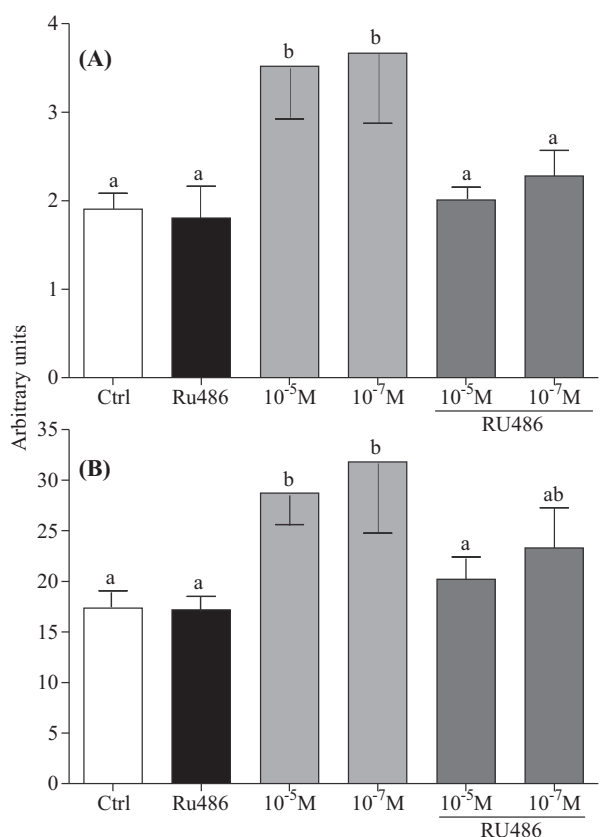


Fig. 8. Influence of RU 486 (1×10^{-5} M) on the cortisol-stimulated (1×10^{-5} , 1×10^{-7} M) mRNA expression for NP-I/OT (A) and PGA (B) in luteal cells ($n = 4$ cows) from days 11-15 of the oestrous cycle after 4 h of incubation. Bars with different superscript letters are significantly different ($P < 0.05$).

centrations of E_2 increased OT secretion from bovine granulosa cells (Voss and Fortune 1993), which could suggest involvement of the estradiol receptor (ER) in OT synthesis and secretion. However, a re-

sponse element for ER was not found in the bovine NP-I/OT gene, only its half-palindrome sequence (Adan et al. 1991). In contrast, the increase in NP-I/OT and PGA mRNA expression was blocked by RU 486, evidence of glucocorticoid receptor involvement in the synthesis and secretion of OT. However, there is also no response element for GR in the NP-I/OT gene, which excludes its direct participation in this mechanism. Note that in addition to GR, there are two orphan nuclear receptors involved in the regulation of bovine NP-I/OT expression: Steroidogenic Factor-1 (SF-1, NR5A1) and Chicken Ovalbumin Upstream Promoter-Transcription Factor I (COUP-TFI, NR2F1) (Wehrenberg et al. 1994, Lioutas et al. 1997). The response elements for SF-1 and COUP-TFI in the NP-I/OT gene overlap each other. When ligand-activated GR dimerises with COUP-TFI, the response element for SF-1 can bind with its orphan receptor and resume transcription of NP-I/OT (De Martino et al. 2004, Młynarczyk and Rękawiecki 2010). SF-1 is a transcription factor for genes encoding enzymes involved in steroidogenesis, including 3β -HSD and aromatase P450 (Michael et al. 1995, Leers-Sucheta et al. 1997), which may explain the increase in P_4 and E_2 secretion with cortisol. However, to determine the pathway that involves the interaction of these receptors requires further research.

In conclusion, GCs acting by GRs stimulate the mRNA expression for NP-I/OT and PGA in granulosa and luteal cells. In this way, they can interfere with numerous loops and processes during the oestrous cycle in the female reproductive system.

Acknowledgments

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