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Possible Roles of Intracellular Cyclic AMP, Protein Kinase C and Calcium Ion in the Apoptotic Signaling Pathway in Bovine Luteal Cells

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Abstract. Structural luteolysis occurs by apoptosis of luteal cells. The present study examined the effects of activators of well-characterized second messengers on *Fas* and *caspase-3* mRNA expression and on P4 production in luteal cells in order to trace the pro- and anti-apoptotic factors in the bovine corpus luteum (CL). Cultured bovine mid luteal cells were treated for 24 h with a cyclic AMP analogue (8-bromo cyclic AMP; 8br-cAMP; 2.5 mM), a protein kinase C (PKC) activator (phorbol 12-myristate 13-acetate; PMA; 10 μ M), or calcium ionophore (A23187; 10 μ M). *Fas* and *caspase-3* mRNA expression was inhibited by 8br-cAMP and PMA but was increased by A23187 ($P < 0.05$). In addition, P4 production by bovine luteal cells was stimulated by 8br-cAMP and PMA, whereas it was inhibited by A23187, compared with untreated controls ($P < 0.05$). The overall results suggest that cAMP and PKC suppress apoptosis in bovine luteal cells through inhibition of *Fas* and *caspase-3* mRNA expression and through stimulation of P4 production. Therefore, substances that activate cAMP or PKC may act as survival factors in the bovine CL. Furthermore, substances that mobilize Ca^{2+} may act as apoptotic factors by stimulating *Fas* and *caspase-3* expression in the bovine luteal cells.

Key words: Corpus luteum, Apoptosis, Anti-apoptotic factor, Second messenger, Cow

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In mammals, luteolysis occurs on two levels corresponding to functional luteolysis and structural luteolysis [1]. Structural luteolysis occurs by luteal cell apoptosis [1], which is known to be regulated by Fas antigen (Fas) [2–4] and caspases [3–6]. Fas is a member of the tumor necrosis factor receptor superfamily, and engagement of Fas with Fas ligand (Fas L) induces apoptosis [7]. Caspases, a family of aspartic acid-specific cysteine proteases, are pivotal mediators of apoptosis during regression of the corpus luteum (CL) [5, 8]. Caspase-3 appears to have an especially

important role in luteolysis [3, 5, 9]. Recently, we found that progesterone (P4) protects bovine luteal cells from apoptosis by attenuating *Fas* mRNA expression and caspase-3 activity [3], suggesting that the CL is protected from apoptosis by P4.

Luteinizing hormone (LH) is known to be the major hormonal regulator of P4 synthesis in luteal cells. Cyclic AMP (cAMP) is considered to be a primary second messenger of LH action in the corpus luteum [10, 11]. Two other secondary messengers involved in P4 production are protein kinase C (PKC) and intracellular calcium ions (Ca^{2+}), which are required for the activation of PKC [12]. However, the effects of these factors on apoptosis signaling pathways in bovine luteal cells

remain unknown.

The present study examined the effects of a cAMP analogue, a PKC activator and calcium ionophore on *Fas* and *caspase-3* mRNA expression and on P4 production in cultured bovine luteal cells, in order to trace pro-apoptotic and anti-apoptotic factors in the bovine CL.

Materials and Methods

Collection of CL

Ovaries with a mid-stage CL were collected from Holstein cows at a local abattoir within 10–20 min after exsanguination. Mid-stage CLs were identified by macroscopic observation of the ovary and uterus as described previously [13, 14]. For cell culture experiments, ovaries with CL were submerged in ice-cold physiological saline and transported to the laboratory.

Cell isolation

Luteal cells were prepared by enzymatically dissociating luteal tissue and were cultured as described previously [15]. The luteal cells were suspended in a culture medium, Dulbecco's Modified Eagle's Medium and Ham's F-12 Medium (D/F medium; 1:1 [v/v], D8900; Sigma-Aldrich, Inc., St. Louis, MO, USA) containing 5% calf serum (CS; 16170-078; Gibco BRL, Grand Island, NY, USA) and 20 µg/ml gentamicin (15750-060; Gibco). Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells in the cell suspension consisted of about 70% small luteal cells, 20% large luteal cells, 10% endothelial cells or fibrocytes, and no erythrocytes.

Cell culture and experiments

The dispersed luteal cells were seeded at 2.0×10^5 viable cells in 1.0 ml, in 24-well culture dishes (3524; Costar, Cambridge, MA, USA). After 24 h of culture, the medium was replaced with D/F medium containing 0.1% bovine serum albumin (BSA; A7888; Sigma), 5 ng/ml sodium selenite, and 5 µg/ml transferrin. The cells were then treated with 2.5 mM 8-bromo cyclic AMP (a cyclic AMP analogue; 8br-cAMP; B7880; Sigma), 10 µM A23187 (calcium ionophore; C7522; Sigma), or 10 µM phorbol 12-myristate 13-acetate (a PKC activator; PMA; P8139; Sigma) for 24 h. The conditioned media from the last 24 h of culture were collected

and stored at –30 °C until assayed for P4. For determination of *Fas* and *caspase-3* mRNA expression, total RNA was prepared from the cultured luteal cells using TRIZOL Reagent (15596; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (1 µg) was reverse transcribed using a ThermoScript™ RT-PCR System (11146-016; Invitrogen), and the reaction mixture was used in each PCR together with the appropriate oligonucleotide primer pairs. RT-PCR was carried out using the housekeeping gene *β-actin* as an internal standard. The sequences of the *Fas* primers, which were based on the report of Vickers *et al.* [16], were 5'-ATG GGC TAG AAG TGG AAC AAA AC-3' (5' primer, 23 mer) and 5'-CAG GAG GGC CCA TAA ACT GTT TGC-3' (3' primer, 24 mer). These primers generated a specific 206 base pair (bp) product. The primers for *caspase-3* were based on the ovine *caspase-3* (AF068837) sequence [5]. The primers were chosen using the Primer3 online application (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and were as follows: *caspase-3* forward 5'-AGC AAA CCT CAG GGA AAC CT-3' and reverse 5'-GGC AGG CCT GAA TAA TGA AA-3' (278 bp). The sequences of the *β-actin* primers, which were designed as described by Leung *et al.* [17], were 5'-GAA GAT CTG GCA CCA CAC-3' (5' primer, 18 mer) and 5'-AGA GGC GTA CAG GGA CAG C-3' (3' primer, 19 mer). These primers generated a specific 200 bp product. *Caspase-3* mRNA expression was calculated using these primers. The sequences of the other *β-actin* primers, which were designed as described by Asselin and Fortier [18], were 5'-GAG GAT CTT CAT GAG GTA GTC TGT CAG GTC-3' (5' primer, 30 mer) and 5'-CAA CTG GGA CGA CAT GGA GAA GAT CTG GCA-3' (3' primer, 30 mer). These primers generated a specific 349 bp product. *Fas* mRNA expression was calculated using these primers. The PCRs were carried out using TaKaRa Taq (R001A; Takara Bio Inc., Ohtsu, Shiga, Japan) and a thermal cycler (iCycler Thermal Cycler 170–8720; Bio-Rad, Hercules, CA, USA). The conditions for the PCRs were as follows: after activation of the DNA polymerase by incubating for 0.5 min at 95 °C, 27 (*Fas* and *caspase-3*) and 18 (*β-actin*) cycles of

Fig. 1. Effects of 8-bromo cyclic AMP (8br-cAMP; 2.5 mM), phorbol 12-myristate 13-acetate (a PKC activator; PMA; 10 μ M), and A23187 (calcium ionophore; 10 μ M) on *Fas* mRNA expression in cultured bovine luteal cells (n=4). The cells were treated with 8br-cAMP, A23187, or PMA in the final 24 h of culture. Upper panel: representative sample of specific RT-PCR products for *Fas* (206 bp) and β -actin (349 bp) separated by agarose gel electrophoresis. Lower panel: relative levels of *Fas* mRNA (RT-PCR 27 cycles, arbitrary units). All values are means $\pm$ SEM of densitometric analysis of *Fas* mRNA levels in the cells (relative to β -actin mRNA levels). Different letters indicate significant differences ($P < 0.05$), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

Fig. 2. Effects of 8br-cAMP (2.5 mM), PMA (10 μ M), and A23187 (10 μ M) on *caspase-3* mRNA expression in cultured bovine luteal cells (n=4). The cells were treated with 8br-cAMP, A23187, or PMA in the final 24 h of culture. Upper panel: representative sample of specific RT-PCR products for *caspase-3* (278 bp) and β -actin (200 bp) separated by agarose gel electrophoresis. Lower panel: relative levels of *caspase-3* mRNA (RT-PCR 27 cycles, arbitrary units). All values are means $\pm$ SEM of densitometric analysis of *caspase-3* mRNA levels (relative to β -actin mRNA levels). Different letters indicate significant differences ($P < 0.05$), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

reactions including denaturation for 0.5 min at 95 C, annealing for 1 min at 60 C, and extension for 1 min at 70 C were performed, followed by an additional extension for 5 min at 72 C. The PCR amplification was calibrated to determine the optimal number of cycles that would allow detection of the appropriate mRNA transcripts while still keeping amplification of these genes in the log phase. A portion (40%) of each reaction mixture was electrophoresed on a 1.5% agarose gel with a known standard (100 bp Ladder; N3231S; New England BioLabs Inc., Beverly, MA, USA), stained with ethidium bromide, and photographed under UV illumination. The relative band intensities were analyzed by computerized densitometry using NIH Image (National Institutes of Health, Bethesda, MD, USA).

P4 determination

The concentrations of P4 were determined directly from the cell culture media using a previously described enzyme immunoassay [19], which was modified from its original method [20].

Briefly, progesterone 3-(O-carboxymethyl) oxime linked to peroxidase type VI through a 6-aminocaproic acid spacer (P3659; Sigma) was used as a tracer (1:10,000 final dilution). Antiserum of progesterone (OK-1) was produced as follows: 11 α -hydroxyprogesterone-11-hemisuccinate-BSA (H4508; Sigma) was suspended in physiological saline (0.25 ml), emulsified with complete Freund's adjuvant (0.25 ml; Difco Laboratories, Detroit, MI, USA), and then injected intradermally into a female rabbit (New Zealand White) at a number of sites on her back and abdomen. After 20 injections at 2- to 4-wk intervals, blood samples were taken. Cross-reactivity of the anti-progesterone serum, validated

Fig. 3. Effects of 8br-cAMP (2.5 mM), PMA (10 μ M), and A23187 (10 μ M) on progesterone production in bovine luteal cells (mean $\pm$ SEM, $n=4$). The cells were treated with 8br-cAMP, A23187, and PMA in the final 24 h of culture. All values are expressed as a percentage of the control value. The concentration of progesterone in the control was 889.89 ± 36.6 ng/ml. Different letters indicate significant differences ($P<0.05$), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

by comparing the inhibition of binding of peroxidase-labeled progesterone to antiserum, were as follows: progesterone, 100%; 5 α -pregnenedion, 7.1%; pregnenolone, 2.4%; 17 α -hydroxyprogesterone, 1.0%; 20 α -hydroxyprogesterone, 0.7%; deoxycorticosterone, 1.2%; corticosterone, 0.3%; estradiol-17 β , <0.1%; testosterone, <0.1%; and androstenedione, <0.1%. The final antibody dilution was 1:100,000. The standard curve ranged from 0.391 to 100 ng/ml, and the effective dose of the assay for 50% inhibition (ED50) was 4.5 ng/ml. The intra- and inter-assay coefficients of variation were 9.3 and 14.5%, respectively.

Statistical analysis

All experimental data are shown as the mean $\pm$ SEM. The data on the effects of 8br-cAMP, A23187, and PMA on P4 production are shown as a percentage of the control. The statistical significance of differences in P4 concentrations and *Fas* and *caspase-3* mRNA expression were assessed by analysis of variance (ANOVA) followed by a Fisher's protected least-significant difference (PLSD) procedure as a multiple comparison test.

Results

Effects of 8br-cAMP, PMA, and A23187 on Fas and caspase-3 mRNA expression

Representative samples of the *Fas*- and *caspase-3*-specific RT-PCR products (206 and 278 bp) are shown in the upper panels of Figs. 1 and 2, respectively. The relative signal intensities for PCR products specific for *Fas* and *caspase-3* mRNAs were assessed after correction based on the β -actin signal intensities (lower panels of Figs. 1 and 2, respectively). Both 8br-cAMP and PMA significantly decreased *Fas* mRNA expression ($P<0.05$), while A23187 increased *Fas* mRNA expression ($P<0.05$). Similar to *Fas*, the mRNA expression of *caspase-3* was significantly decreased by 8br-cAMP and PMA ($P<0.05$) whereas it was increased by A23187 ($P<0.05$).

Effects of 8br-cAMP, PMA, and A23187 on P4 production

P4 production by bovine luteal cells was stimulated by 8br-cAMP and PMA, whereas it was inhibited by A23187, compared with untreated controls ($P<0.05$; Fig. 3).

Discussion

The preceding results demonstrate that well-known second messengers (cAMP, PKC, and Ca^{2+}) are related to apoptosis in bovine luteal cells through regulation of *caspase-3* and *Fas* mRNA expression. *Fas* is a cell surface receptor that triggers apoptosis in sensitive cells when *Fas* L binds to it [7], whereas *caspase-3* is a member of the caspase family, which has pivotal roles in apoptosis in many cell types, including bovine luteal cells [3, 5, 8]. The roles of *Fas* and *caspase-3* in luteal regression have previously been documented in cattle [2, 5, 6]. In the present study, the findings that *Fas* and *caspase-3* mRNA expression was suppressed by 8br-cAMP and PMA indicate that cAMP accumulation and activation of PKC play roles in protecting bovine luteal cells from apoptosis.

CL function is regulated by various bioactive substances, such as gonadotropins, steroids, and growth factors. Among these substances, luteinizing hormone (LH) [21, 22], epidermal growth factor (EGF) [23], and prostaglandin (PG)

E2 [24, 25] have been reported to stimulate accumulation of cAMP via activation of adenylate cyclase (AC), whereas PGF2 α is known to activate PKC in luteal cells [15, 26]. Therefore, it is possible that substances that activate AC or PKC, such as LH, EGF, PGE2, and PGF2 α may act as survival factors and protect the bovine CL against apoptosis. Further studies are needed to clarify which substances have anti-apoptotic roles in the bovine CL.

We have recently shown that P4 suppresses apoptosis in bovine luteal cells through inhibition of the expression of apoptosis-related genes and by the suppression of caspase-3 activity [3]. In the present study, 8br-cAMP and PMA stimulated P4 production, whereas they inhibited *Fas* and *caspase-3* mRNA expression in luteal cells. Thus, the suppression of *caspase-3* and *Fas* mRNA expression by 8br-cAMP and PMA observed in the present study might be mediated by P4. In support of this idea, we previously found that *Fas* [2] and *caspase-3* mRNA expression was low in the bovine CL in the mid and late luteal stages when the CL synthesizes high amounts of P4 (unpublished data).

On the other hand, we demonstrated that inducing intracellular Ca²⁺ mobilization by A23187 suppressed P4 production, whereas Ca²⁺ increased *Fas* and *caspase-3* mRNA expression in the luteal

cells in the present study. The vasoactive peptides endothelin-1 (ET-1) and angiotensin II (Ang II) act through Ca²⁺ mobilization [27, 28]. In addition, these peptides have been suggested to play important roles during physiological and PGF2 α -induced luteolysis concomitant with decreasing P4 production in cows [27, 29–31]. Therefore, ET-1 and Ang II might be the factors that induce apoptosis via Ca²⁺ mobilization in bovine luteal cells.

The overall results of the present study indicate that the intracellular signaling pathways via cAMP, PKC, or Ca²⁺ are related to the regulation of luteal cell apoptosis and suggest that the substances that act via cAMP or PKC protect cells from apoptosis, whereas those that act via Ca²⁺ induce apoptosis in bovine luteal cells.

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