Use of protein kinase C and phospholipase A2 inhibitors in bovine endometrial cells treated with estradiol and calcium ionophore[*]

Uso de inibidores de proteína quinase C e fosfolipase A2 em células endometriais bovinas tratadas com estradiol e ionóforo de cálcio

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ABSTRACT

The release of endometrial prostaglandin-F2α (PGF2α) in bovine females can be induced *in vivo* by estradiol (E_2). However, its role in this mechanism has not been clarified. We hypothesized that E_2 stimulates the activity and abundance of protein kinase C (PKC) and phospholipase A2 (PLA2). Our objective in this study was to analyze the effects of PKC and PLA2 inhibitors on PGF2α synthesis induced by E_2 and calcium ionophore (CI) in bovine endometrial cells (BEND cells; Experiment 1). Additionally, we evaluated the abundance of PKC and PLA2 in endometrial explants of cows treated or not with E_2 17 days after estrus (D17, D0 = estrus; Experiment 2). In Experiment 1, BEND cells were submitted to a PKC inhibitor (10 μM of $C_{25}H_{24}N_4O_2$; bisindolylmaleimide I, or BIS I), a PLA2 inhibitor (20 μM of arachydoniltrifluoromethane or AACOCF3), or none. The BEND cells were subsequently treated with E_2 and CI, and PGF2α concentrations were measured in the culture medium through radioimmunoassay. For DIF-12 (PGF2α concentration 12 h after treatment subtracted from PGF2α concentration at hour 0), no PKC inhibitor effect was observed ($P= 0.2709$). However, DIF-12 was lower ($P < 0.05$) for groups treated with the PLA2 inhibitor and PLA2 inhibitor + CI + E_2 groups than the control and CI + E_2 groups. Thus, AACOCF3 was an efficient PLA2 inhibitor in the BEND cells culture system, and E_2 did not stimulate the synthesis of PKC and PLA2. In Experiment 2, cyclic Nellore heifers received none (n = 5) or 3 mg (n = 6) of 17β-E_2 on D17 and were slaughtered 2 h after administration. The abundance of PKC and PLA2 in the endometrial tissue was evaluated using Western blotting analysis. No E_2 effect was observed on PKC (P = 0.08) and PLA2 (P = 0.56). We concluded that E_2 did not stimulate the activity and abundance of PKC and PLA2.

Keywords: Estradiol. PGF2α. PKC. PLA2. BEND cells.

RESUMO

A liberação endometrial de prostaglandina-F2α (PGF2α) em fêmeas bovinas pode ser induzida *in vivo* pelo estradiol (E_2). Entretanto o seu mecanismo de ação ainda não foi bem esclarecido. Nossa hipótese é que o E_2 estimula a atividade e a abundância da proteína quinase C (PKC) e da fosfolipase A2 (PLA2). Nosso objetivo com este estudo foi analisar os efeitos de inibidores de PKC e PLA2 na síntese de PGF2α induzida por E_2 e ionóforo de cálcio (CI) em células endometriais bovinas (células BEND; Experimento 1). Adicionalmente, nós avaliamos a abundância de PKC e PLA2 em explantes endometriais de vacas tratadas com ou sem E_2 17 dias após o estro (D17, D0 = estro; Experimento 2). No Experimento 1, células BEND foram submetidas ao inibidor de PKC (10 μM de $C_{25}H_{24}N_4O_2$; bisindolylmaleimide I, ou BIS I), e ao inibidor de PLA2 (20 μM de arachydoniltrifluoromethane ou AACOCF3) ou a nenhum inibidor. As células BEND foram subsequentemente tratadas com E_2 e CI e concentrações de PGF2α foram mensuradas no meio de cultura por radioimunoensaio. Para DIF-12 (concentração de PGF2α 12 horas depois do tratamento, subtraída da concentração de

PGF2α na hora 0), não foi observado efeito do inibidor de PKC (P = 0.2709). Entretanto DIF-12 foi menor ($P < 0.05$) nos grupos tratados com inibidor de PLA2 e inibidor de PLA2 + CI + E_2 quando comparados com o grupo controle e o grupo CI + E_2. O AACOCF3 foi um eficiente inibidor de PLA2 em sistema de cultura de células BEND e o E_2 não estimulou a síntese de PKC e PLA2. No Experimento 2, novilhas Nelore cíclicas receberam 3 mg de 17β-E_2 (n = 6) ou nenhum tratamento (n = 5) no D17 e foram abatidas duas horas depois da administração dos tratamentos. A quantidade de PKC and PLA2 no tecido endometrial foi avaliada pela técnica de Western Blotting. Não foi observado efeito do E_2 sobre a PKC ($P= 0.08$) e nem sobre a PLA2 ($P= 0.56$). Conclui-se que o E_2 não estimulou a atividade e abundância de PKC e PLA2.
Palavras-chave: Estradiol. PGF2α. PKC. PLA2. Células BEND.

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Introduction

Prostaglandin-F2α (PGF2α) is the primary luteolytic agent in bovine females, promoting luteolysis (Carambula et al., 2002). The stimulation of PGF2α synthesis involves endocrine factors such as estradiol (E_2), oxytocin (OT), progesterone (P4), and luteinizing hormone. The suppression of E_2, through irradiation or cauterization of ovarian follicles, delays luteolysis (Hughes et al., 1987), whereas the administration of E_2 in cyclic cows stimulates it (Thatcher et al., 1986). In this context, E_2 may play a crucial role in luteolysis. However, the mechanisms by which E_2 performs this function have not been sufficiently clarified.

The synthesis of PGF2α results from a cascade of highly coordinated intracellular events involving the sequential activation of several proteins – guanine nucleotide-binding proteins (G proteins), protein kinase C (PKC), phospholipases A2 and C (PLA2 and PLC), and cyclooxygenase 2 (COX-2), among others. Burns et al. (1997) described a model for PGF2α synthesis in epithelial cells of the endometrium. In their model, OT binds to its receptors, which, in association with G proteins, activate PLC that cleaves phosphatidylinositol bisphosphate (PIP2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to receptors in the endoplasmic reticulum, promoting the release of endoplasmic calcium to the cytosol. DAG activates PKC, also dependent on calcium (Alberts et al., 1997). The activated PKC phosphorylates cytosolic PLA2 (cPLA2) into PLA2, which cleaves phosphatidylcholine, releasing arachidonic acid (Gijon & Leslie, 1999). Lastly, the free arachidonic acid is converted by COX-2 into prostaglandin-H_2, which, through the action of the prostaglandin-F synthase – in particular, aldose reductases AKR1B1 and AKR1C3 – is converted into PGF2α (Fortier et al., 2008).

Membrive et al. (2014) cited an increased in PGF2α synthesis in cows treated with E_2 *in vivo* at 17 days after estrus in association with calcium ionophore (CI) added *in vitro* to their endometrial explants. Using the same treatment, they observed a 179% increase in PGF2α production when CI was used by itself compared to the control group. However, when CI was used in association with E_2, the increase was 340% compared to the control group, and 58% compared to the CI- group only.

Thus, we hypothesized that E_2 stimulates the activity and abundance of calcium-dependent enzymes, specifically PKC and PLA2. Based on this, the objective of Experiment 1 was to evaluate the effect of PKC and PLA2 inhibitors on PGF2α synthesis induced by E_2 and CI in bovine endometrial BEND cells. And Experiment 2 was developed to determine the abundance of PKC and PLA2 proteins in endometrial explants of cows treated or not with E_2 17 days after estrus (D17, D0 = estrus).

Material and Methods

Experiments location

The experiments were carried out at the University of São Paulo (USP), at the College of Veterinary Medicine and Animal Sciences (FMVZ), at the Center of Biotechnology in Animal Reproduction, Laboratory of Physiology, and Molecular Endocrinology (LFEM), Pirassununga, São Paulo, Brazil. All procedures performed have been reviewed

and approved by the Ethical Committee for Animal Use in Research, FMVZ - USP (protocol number 1.861/2009).

Experiment 1

BEND cell cultures

We obtained BEND cells (Staggs et al., 1998) from the American Type Culture Collection (ATCC; CRL-2398, USA). The cells were suspended in a complete culture medium with serum containing: 40% Ham's F-12 nutrient mixture (Sigma N6760); 40% minimal essential medium (MEM; Sigma M0643); 200 IU insulin/L (Sigma I5500); 10% v/v fetal bovine serum (FBS; Gibco Life 10270-106); 10% v/v equine serum (Nutricel); and 1% v/v antibiotic and antimycotic solution (Sigma A7292) (Membrive et al., 2014). The BEND cells were plated in 24-well plates (4×10^4 cells/well; Corning Incorporated) with 1.5 cm in diameter and 2 cm in height, where 1.5 mL of complete culture medium was added in each well. They were cultivated at 38.5°C in a humidified atmosphere containing 5% CO_2 until they reached the confluence of 90%. The cells were then washed twice in a culture medium without fetal bovine and equine serum (referred to as a serum-free medium). Following another two consecutive washes, the cells were incubated in serum-free medium for 24 h, after which the wells of the plates containing the cultured cells were evenly divided to receive the different treatments. To better understand the experiments carried out, they were called Experiment 1A (PKC inhibitor) and Experiment 1B (PLA2 inhibitor).

Experiment 1A

The PKC protein inhibitor $C_{25}H_{24}N_4O_2$ (bisindolylmaleimide I or BIS I; Calbiochem 203290) was initially diluted in dimethyl sulfoxide at an initial concentration of 4.125 mg/mL. Confluent cells were divided into four treatment groups: serum-free medium (control group); serum-free medium with 10 μM BIS I (PKC inhibitor group); serum-free medium with 10 μM BIS I, 10^{-6} M CI (A23187), and 10^{-13} M 17β-E_2 (Sigma E-8875; PKC inhibitor + CI + E_2 group); and serum-free medium with 10^{-6} M of CI and 10^{-13} M of 17β-E_2 (CI + E_2 group). The cells were incubated for 60 min, with 1 mL of serum-free medium alone or containing the inhibitors designated in each treatment. After this period, a 100 μL sample of the medium was collected (Time 0; T0). Immediately afterward, 100 μL of serum-free medium alone or containing E_2 + CI was added to the remaining 900 μL in the wells, for a final concentration of 10^{-6} M CI and 10^{-13} M E_2. Samples containing 100 μL of the medium were collected 12 h after treatment (T12). Each experiment was repeated three times. In each repetition, three wells were used, in a total of nine wells per treatment. The samples were stored at –20°C for later measurement of PGF2α by radioimmunoassay.

Experiment 1B

The PLA2 protein inhibitor arachydoniltrifluoromethane (AACOCF3; Calbiochem 100109) was diluted in ethanol at the initial concentration of 6.5 mg/mL. The confluent cells were divided into the following groups: serum-free medium (control group); serum-free medium with 20 μM of AACOCF3 (PLA2 inhibitor group); serum-free medium with 20 μM AACOCF3, 10^{-6} M CI, and 10^{-13} M 17β-E_2 (PLA2 inhibitor + CI + E_2 group); and serum-free medium with 10^{-6} M CI and 10^{-13} M 17β-E_2 (CI + E_2 group). The cells were incubated for 60 min with 1 mL of serum-free medium initially containing only the inhibitors designated in each treatment. After this period, a 100 μL sample of the medium was collected (T0). Immediately afterward, 100 μL of serum-free medium alone or containing E_2 + CI was added to the remaining 900 μL in the wells for a final concentration of 10^{-6} M CI and 10^{-13} M E_2. Samples containing 100 μL of the medium were collected at T12. Each experiment was repeated three times. In each repetition, three wells were used, totaling nine wells per treatment. The samples were stored at -20 °C for later measurement of PGF2α by radioimmunoassay.

Radioimmunoassay

For this experiment, the technique used was the one described by Danet-Desnoyers et al. (1995) to measure the PGF2α concentrations collected in the culture mediums. Three assays were performed for this purpose. The intra-assay coefficients of variation for the reference values i) 250 pg/mL of PGF2α were 8.58%, 6.93%, and 8.67%; ii) 1,000 pg/mL of PGF2α were 4.85%, 6.07%, and 8.37%; and iii) 3,500 pg/mL of PGF2α were 7.86%, 19.27%, and 7.52%. The inter-assay coefficients of variation for the reference values 250, 1,000, and 3,500 pg/mL of PGF2α were 8.52%, 5.24%, and 13.25%, respectively.

Statistical Analysis

The dependent variable was DIF-12, which was obtained by subtracting the PGF2α concentrations at T12 from the PGF2α concentrations at T0. The independent variables were repetition, treatment, and repetition x treatment interaction. A preliminary analysis demonstrated that the production data of PGF2α (DIF-12) were adequate regarding the homogeneity of the variances (Test F, $P >$

0.05), which were presented using LSmeans + EPM. The data were analyzed with Analysis of Variance (ANOVA) using the PROC GLM of the SAS software. Orthogonal contrasts were used for comparing the treatments.

Experiment 2

Animals

For this experiment, a total of 11 Nellore heifers (*Bos taurus indicus*) were used, of variable age, non-pregnant, non-lactating, cyclical, and with a body condition score between 5 and 6 (on a scale of 1 to 9). The animals were kept in paddocks with free water access, and their diet was based on grazing (*Brachiaria spp.*) and supplemented with minerals. All animals were dewormed, vaccinated, and subjected to tuberculosis and Brucellosis control tests before this study. The Ethical Committee for Animal Use in Research, FCAT - UNESP, previously reviewed and approved the procedures performed (protocol number 31/2018.R1).

Ovulation synchronization protocol

For ovulation synchronization, all females were subjected to the same hormonal protocol. Ten days before D0 (D -10), heifers received an intravaginal device containing 1 g of P4 (Cronipress Monoset®, Biogenesis Bago) associated with 2 mg of E_2 Benzoate (Gonadiol®, Zoetis) intramuscularly (IM). Six days later (D -4), they received an injection of 150 μg IM of D-cloprostenol (Preloban®, Intervet). After 48 h (D -2), the P4 devices were removed, and the heifers received another injection of 150 μg IM of D-cloprostenol. On what we considered to be D0 (48 h after removing the devices), an ultrasound examination was performed (Aloka, model SSD500, 7.5 MHz linear transducers). The females who had a dominant follicle bigger than 7.5 mm received a 100 μg IM injection of gonadorelin, a synthetic gonadotropin-releasing hormone (GnRH; Fertagil®, Intervet) on D0, to induce ovulation. A new ultrasound exam was performed on D2 (48 h after the GnRH injection) to evaluate which females had ovulated. The heifers who participated in this study had a dominant follicle bigger than 7.5 mm in D0 and whose ovulation was confirmed in D2. On D6, another ultrasound examination was performed. Heifers that had a dominant follicle bigger than 7.5 mm then received a 100 μg injection IM of gonadorelin to induce ovulation and promote the formation of an accessory corpus luteum. A new exam was performed on D8 (48 h after the second GnRH injection) to evaluate the females that ovulated. In the present study, we used heifers in which ovulation was confirmed on D8, and that had formed an accessory corpus luteum.

Quantification of P4 concentrations by radioimmunoassay (RIA)

All animals used in this study had plasma P4 concentrations over 1 ng/mL on D17. Additionally, the analyses were performed on animals that had not started physiological luteolysis at the time of slaughter (D17), considering animals that had P4 concentration ≥ 50% of the previous day. The plasma P4 concentrations were assayed with a solid-phase RIA kit containing antibody-coated tubes and I^{125}-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA). The assay sensitivity was 0.03 ng/mL. Samples containing standard high (HC) and low concentrations (LC) of P4 determined the intra- and inter-assays variations. The intra-assay variations for HC and LC were 6.72% and 0.29%, respectively.

Treatments

On D17, intravenously injection with 6 mL of a solution with 50% ethanol (control group; n = 5) or 3 mg of 17β-E_2 diluted to 6 mL of a solution with 50% ethanol (E_2 group; n = 6) was injected in the animals The heifers were slaughtered 2 h after treatment. Slaughter was carried out by cerebral concussion with a pneumatic pistol.

Isolation and storage of endometrial explants

The endometrium of the uterine horn ipsilateral to the original corpus luteum (non-accessory) was dissected and the intercaruncular region's fragments were packed in cryotubes and immediately stored at –80ºC.

Protein extraction and quantification

To each 2 g of endometrium were added 2.5 mL of a protein extraction solution containing 1 mM EDTA (Fisher BP 120-500), 1 mM EGTA (Sigma E8145), 1 mM DTT (Fisher BP 172-5), 0.5 mM PMSF (Sigma P-7626), 300 mM NaCl, 0.5% NP-40, 50 mM Tris pH 8.0, 10% Glycerol, 10 μg/mL Aprotinin (Sigma A-4529), 10 μg/mL Leupeptin (Sigma L-2884), and 10 μg/mL Pepstatin (Sigma P-4265). Proteins were quantified using the classic Bradford method.

Western blotting

We evaluated the measures of the control (n = 5) and E_2 groups (n = 6). As the samples were not applied to gel in triplicate, each gel was repeated in triplicate, totaling nine runs for each sample. We used separating gels with 12% Polyacrylamide. To make the gels, we used the Kaleidoscope

Prestained Molecular Weight Standards (PPM; cat. 161-0324, BIO-RAD), which contained proteins with the following molecular weights: 193.89, 120.37, 88.95, 36.26, 31.04, 16.38, and 6.62 kDa. The total protein administered to each well of the gel amounted to 50 μg. Each tissue sample was submitted to an electrophoretic run in duplicate. The same sample was placed on both gel runs, in the same sequence, so that each run was obtained with two identical gels containing the same samples. This procedure was repeated so that each sample was run in a total of four wells, in quadruplicate, to ensure reliable comparison among the samples and different treatments. The electrophoretic assay was adjusted to 220 V and 100 mA for 90 min. After this established period, the gels were immersed in a transfer buffer containing methanol for 2 h.

We then performed a horizontal transfer, accommodating two blotting papers, a nitrocellulose membrane, and a polyacrylamide gel. The transfer conditions were 47 V and 100 mA for approximately 2 h. At the end of the transfer, the nitrocellulose membranes were immersed in a blocking solution with skimmed milk. They remained there in incubation for 2 h, being subjected to constant agitation on the shaking plate at room temperature. Then, the membranes were washed four times with TBS solution, for 10 min at each wash. After the fourth wash, the membranes were incubated with 1 mL of the TBS Buffer solution containing the diluted primary antibody, at a temperature of 4°C, under a shaking plate, for 12 h. After prior validation, the primary antibodies were used at a dilution of 1:500 of PKC (93775, phospho-PKC theta; T538, rabbit Ab, Cell Signaling) and 1:600 of PLA2 (sc-55887, group V PLA2; T-20, IgG polyclonal goat, Lot. A1508, Santa Cruz Biotechnology). The antibodies were then diluted in TBS Buffer Solution. After incubation with the primary antibodies, the membranes were washed on a shaking platform three times for 10 min (totaling 30 min) with TTBS Buffer Solution and once for 10 min with TBS Buffer Solution. After validation, the secondary antibodies used a dilution of 1:3,000 of anti-PKC (7074, anti-rabbit IgG-HRP linked antibody, Cell Signaling) and 1:10,000 of anti-PLA2 (sc-2020, donkey anti-goat IgG-HRP, Lot. B2709, Santa Cruz Biotechnology). The secondary antibodies were diluted in TBS Buffer Solution. After the last wash of the primary antibodies, the membranes were incubated with 20 mL of the solution containing the secondary antibodies for 60 min, under a shaking plate at room temperature. After incubating the membranes in a solution containing the secondary antibodies (anti-PLA2 and anti-PKC), they were washed three times for 10 min (totaling 30 min) with the TTBS Buffer Solution. The bands were visualized through the ECL Prime Western

Blotting Detection Reagent (GE Amersham; RPN2232, GE Healthcare Life Sciences) and the Chemi Doc™ Imaging System (170-8280; Bio-Rad Laboratories) equipment. The membranes were incubated in this chemiluminescent solution for 3 min for PKC and 1 min for PLA2, times stipulated after previous validation. The optical densities of the images obtained were analyzed using the Image Lab 5.2 software (Bio-Rad Laboratories). Differences among groups were evaluated based on the mean optical density of animals in each group.

Statistical analysis

After the development, the optical density of each specific band correlated positively to its concentration. The detected bands' digital density was analyzed with ANOVA using the PROC GLM of the SAS software. In the model, the dependent variable was the density of each of the bands, and the independent variables were subject and treatment (control and E_2 groups) for the PKC and PLA2. The imposed significance was 95%.

Results

Experiment 1

Experiment 1A

Figure 1 shows the average PGF2α concentrations. At T0, PGF2α concentrations were: i) 460.96 ± 17.54 pg/mL (control group); ii) 513.18 ± 22.32 pg/mL (PKC inhibitor group); iii) 444.93 ± 17.63 pg/mL (PKC inhibitor + CI + E_2 group); and iv) 565.31 ± 20.1 pg/mL (CI + E_2 group). At T12, the averages were: i) 628.1 ± 22.86 pg/mL (control group); ii) 620.01 ± 19.45 pg/mL (PKC inhibitor group); iii) 649.08 ± 21.74 pg/mL (PKC inhibitor + CI + E_2 group); and iv) 805.4 ± 22.41 pg/mL (CI + E_2 group).

The DIF-12 values are illustrated in Figure 2, as: i) 167.16 ± 18.17 pg/mL (control group); ii) 106.82 ± 17.9 pg/mL (PKC inhibitor group); iii) 190.12 ± 14.61 pg/mL (PKC inhibitor + CI + E_2 group); and iv) 235.54 ± 18.61 pg/mL (CI + E_2 group). There was a repetition effect ($P < 0.001$); however, there was no inhibitor effect ($P = 0.2709$). The cells treated with the PKC inhibitor (PKC inhibitor and PKC inhibitor + CI + E_2 groups) did not differ in DIF-12 compared to the groups not treated with the PKC inhibitor (control and CI + E_2 groups).

Experiment 1B

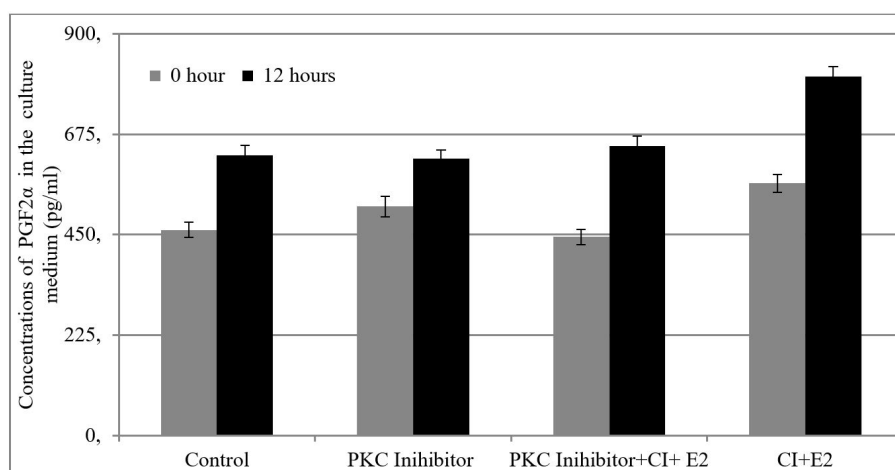In Experiment 1B, the average of PGF2α concentrations are shown in Figure 3. At T0, the concentrations were: i)

Figure 1 - Experiment 1A: Concentrations of PGF2α in the culture medium (pg/mL), at 0 and 12 h, for bovine endometrial cells (BEND) at the Control Group, PKC inhibitor Group, PKC inhibitor + CI + E_2 Group, and CI + E_2 Group.
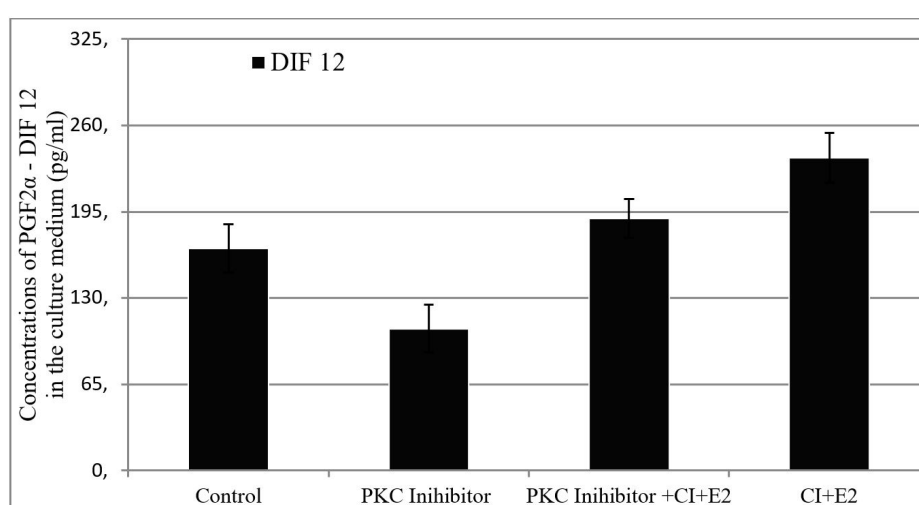

Figure 2 - Experiment 1A: PGF2α concentrations (pg / mL), with DIF between 0 and 12 h, for bovine endometrial cells (BEND) at the Control Group, PKC inhibitor Group, PKC inhibitor + CI + E_2 Group, and CI + E_2 Group.

460.96 ± 17.54 pg/mL (control group); ii) 724.13 ± 20.21 pg/mL (PLA2 inhibitor group); iii) 656.39 ± 20.41 pg/mL (PLA2 inhibitor + CI + E_2 group); and iv) 565.31 ± 20.12 pg/mL (CI + E_2 group). At T12, the averages were: i) 628.1 ± 22.86 pg/mL (control group); ii) 781.28 ± 22.67 pg/mL (PLA2 inhibitor group); iii) 781.81 ± 22.23 pg/mL (PLA2 inhibitor + CI + E_2 group); and iv) 805.4 ± 22.41 pg/mL (CI + E_2 group).

The DIF-12 values are illustrated in Figure 4, as: i) 167.16 ± 18.17 pg/mL (control group); ii) 91.47 ± 17.69 pg/mL (PLA2 inhibitor group); iii) 125.42 ± 14.59 pg/mL (PLA2 inhibitor + CI + E_2 group); and iv) 235.54 ± 18.61 pg/mL (CI + E_2 group). There was a repetition effect ($P < 0.001$) as well as an inhibitor effect ($P < 0.05$). The cells treated with the PLA2 inhibitor (PLA2 inhibitor and PLA2 inhibitor + CI + E2 groups) had a lower DIF-12 ($P < 0.05$) compared to the groups not treated with the PLA2 inhibitor (control and CI + E_2 groups).

Experiment 2

In Experiment 2, the optical density averages of endometrial PKC were 1.0001 ± 0.1869 (control group) and 0.9631 ± 0.1447 (E_2 group). There was no significant effect of E_2 ($P = 0.88$) on PKC increase in endometrial tissue. For PLA2, the averages were 1.0007 ± 0.3253 (control group) and 1.2521 ± 0.2520 (E_2 group). There was no significant effect of E_2 ($P = 0.56$) on PLA2 increase in endometrial tissue.

Discussion

In the present study, the DIF-12 was lower in the PLA2 inhibitor and PLA2 inhibitor + CI + E_2 groups than the control and CI + E_2 groups. Membrive et al. (2014) noted that E_2 increased endometrial cells' sensitivity to CI ($P < 0.01$), while CI associated with E_2 did not promote an increase in PGF2 exposure in BEND cells. However, in our experiments, the use of CI associated with E_2 (PLA2 inhibitor + CI + E_2 and CI + E_2 groups) did not differ
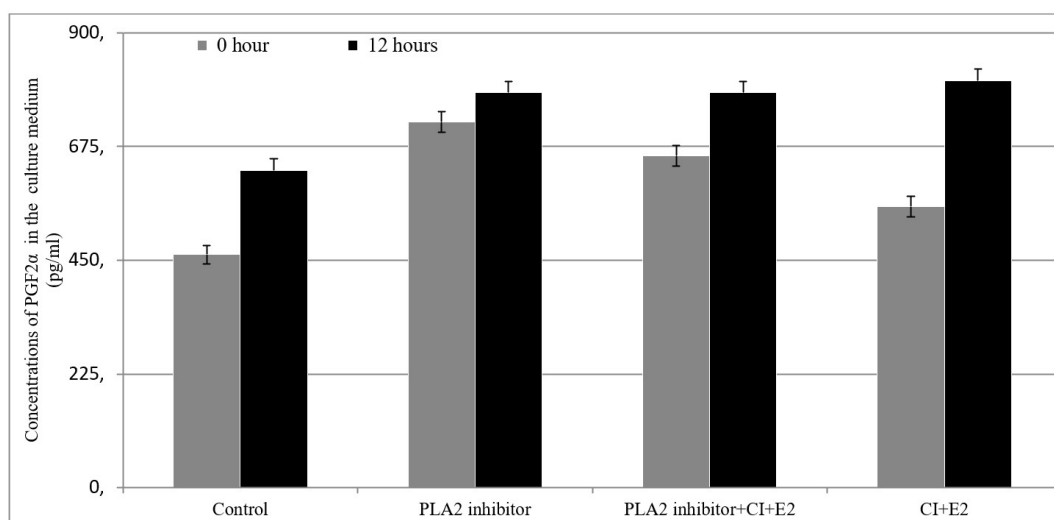
Figure 3 - Experiment 1B: PGF2α concentration (pg / mL), at times 0 and 12 h, for cells at the Control Group, PLA2 inhibitor Group, PLA2 inhibitor + CI + E_2 Group, and CI + E_2 Group.
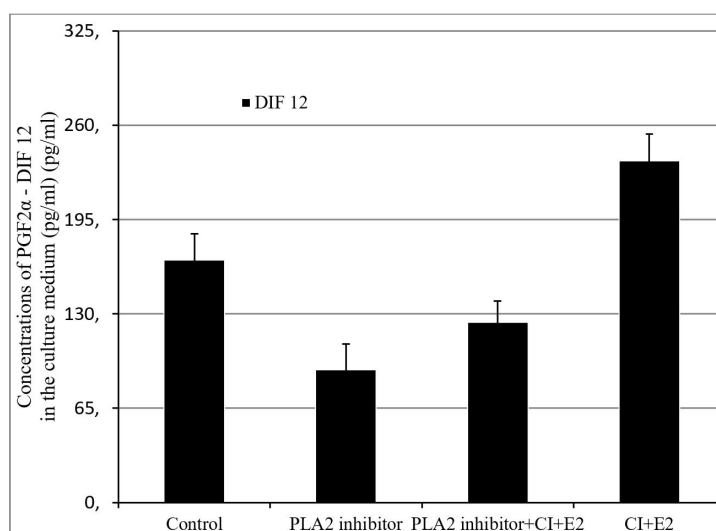

Figure 4 - Experiment 1B: PGF2α concentrations (pg / mL), with DIF between 0 and 12 h, for bovine endometrial cells (BEND) treated with Control Group, PLA2 inhibitor Group, PLA2 inhibitor + CI + E_2 Group, and CI + E_2 Group.

from no treatment (control and PLA2 inhibitor groups) regarding DIF-12 (235.54 ± 18.61 *vs.* 167.16 ± 18.17 pg/mL, respectively; $P > 0.05$).

Ionophores, including CI, are small hydrophobic molecules that dissolve the lipid bilayer of the plasma membrane, which controls permeability and facilitates molecule entry into the cell. CI is an ionic carrier of calcium transporters, which acts by transporting two H^+ ions out of the cell for each calcium ion transported into it. When cells are exposed to CI, the calcium present in the extracellular liquid enters the cytosol due to a strong electrochemical gradient, increasing free calcium in the cytosol. In our experiment, the use of CI probably enabled a more significant entry of calcium in endometrial cells. However, its function as a second messenger may not have been evidenced due to the limited presence of PKC and PLA2, known to have a calcium-dependent activation.

This PKC- and PLA2-shortage is, presumably, because E_2 did not stimulate both enzymes' synthesis, contradicting our initial hypothesis.

Considering that, in Experiment 1, a repetition effect was observed ($P < 0.001$), it was evident that, with each repetition, the results differed in terms of treatment. Thus, in this study specifically, the cell model did not confer repeatability. In the absence of CI stimulation associated with E_2, the search to verify the inhibitors' effects was quite limited. In Experiment 1A, the cells treated with the PKC inhibitor (PKC inhibitor and PKC inhibitor + CI + E_2 groups) did not differ in DIF-12 from the groups not treated with the PKC inhibitor (control and CI + E_2 groups). In this study, PKC inhibition did not limit PGF2α synthesis in BEND cells. In Experiment 1B, the cells treated with the PLA2 inhibitor (PLA2 inhibitor and PLA2 inhibitor + CI + E_2 groups) had lower DIF-12 ($P < 0.05$) than the groups not

treated with the PLA2 inhibitor (control group and CI + E_2 group). Thus, blocking PLA2 activity limits PGF2α synthesis in BEND cells.

Burns et al. (1997) found a considerable increase in PGF2α synthesis when treating endometrial explants with PLA2 stimulators. Uterine E_2 implants in rats were also reported to promote a 167-fold increase in PLA2 activity when compared to the placebo. These findings reinforced our idea that E_2 would potentiate PLA2 activity. Therefore, we hoped that E_2 would increase transcripts from PLA2, potentiating PGF2α synthesis in BEND cells, which we did not observe.

In the present study, the E_2 did not stimulate the synthesis of PKC and PLA2 in beef cattle endometrial tissue, we expected an increase in PKC and PLA2 in endometrial explants from cows treated with E_2 on D17. However, there was no significant effect of E_2 on the increase of either PKC ($P = 0.88$) or PLA2 ($P = 0.56$). Oliveira (2017) treated beef cattle with 17β-E_2 on D15 and evaluated the PKCα, PKCβ, and PLA2G4 concentrations in uterine biopsies. In the biopsies, performed 4 and 7 h after treatment, even less enzymatic expression was observed, which was the opposite of what was expected. In Oliveira (2017), the hypothesis that 17β-E_2 would stimulate was also rejected, which corroborates with our results (in Experiments 1 and 2).

Alves et al. (2011) demonstrated that *in vivo* treatment with 17β-E_2 did not modulate the total protein amount in endometrial explants collected 2 h after slaughter. There was also no modulation in the abundance of proteins with molecular weights similar to PKC (approximately 80 kDa). However, Alves et al. (2011) reported an increase in the number of proteins with molecular weights of 75 to 76 kDa and 108 to 110 kDa. Thus, it is suggested that other proteins, which we did not evaluate, may modulate PGF2α synthesis by stimulating 17β-E_2. Oliveira (2017), besides using an immunohistochemistry technique, found that 17β-E_2 modulated PKC isotypes in opposite ways, increasing PKCγ in the uterine luminal epithelium and, at the same time, decreasing the abundance of transcripts and PKCα. The same author suggests that the different PKC subtypes may regulate PGF2α synthesis in a specific way.

Considering these results, we suggest other possible E_2 mechanisms of action. E_2 receptors are also present on the cell membrane, and, once activated, they trigger cellular responses by non-genomic mechanisms (Ho & Liao, 2002). Razandi et al. (1999) observed that the activated E_2 receptor could activate the c-Jun kinase (JNK) activity.

JNK-activated c-Jun would act on gene transcription. Considering these observations, E_2 could indirectly exert a genomic effect on the cell, not acting as the response element itself in gene transcription, but favoring other response elements' performance. Another possibility for E_2 would be participating in the ERK1/2, JNK/SAPK, and p38 MAPK pathways. E_2 may promote the synthesis or activation of these proteins, which, associated with CI, would activate PKC and PLA2, promoting PGF2α synthesis in bovine endometrium. Further studies are needed to elucidate the mechanisms of action by which E_2 stimulates PGF2α synthesis in bovine endometrial cells.

Conclusion

In the present study, the DIF-12 was lower in the PLA2 inhibitor and PLA2 inhibitor + CI + E_2 groups than the control and CI + E_2 groups. Additionally, E_2 did not stimulate the synthesis of PKC and PLA2 in beef cattle endometrial tissue. We concluded that E_2 did not stimulate the activity and abundance of PKC and PLA2.

Conflict of Interest

The Authors of the paper "Use of protein kinase C and phospholipase A2 inhibitors in bovine endometrial cells treated with estradiol and calcium ionophore", declare that they have no know competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This statement is signed by the corresponding author to indicate agreement that the above information is true and correct.

Ethics Statement

We certify that the proposal entitled "Action of 17B-estradiol on the transcription and synthesis of PKC and PLA2 enzymes involved in the synthesis of endometrial PGF2a in bovine females", registered under number 31 / 2018.R1 - CEUA, under the responsibility of Claudia Maria Bertan Membrive - which involves the production, maintenance or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except humans), for scientific research purposes - is in accordance with the provisions of Law No. 11,794, of October 8 of Decree No. 6,899, of July 15, 2009, and with the rules issued by the National Council for the Control of Animal Experimentation (CONCEA), and was approved by the ETHICS COMMISSION ON THE USE OF ANIMALS - CEUA of the Faculty of Agricultural Sciences and Technological University of UNESP - Dracena Campus, in a meeting of 11/21/2018.

References

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Biologia molecular da célula. 3rd ed. Porto Alegre: Artes Médicas; 1997. 1294 p.

Alves S Jr, Castro F, Soares N, Pereira F, Balieiro J, Munin F, Cesar M, Binelli M, Membrive C. Estradiol-17β altera expressão proteica endometrial em fêmeas bovinas tratadas no 17º dia do ciclo estral. Braz J Vet Res Anim Sci. 2011;48(1):27-37. http://dx.doi.org/10.11606/S1413-95962011000100004.

Burns PD, Graf GA, Hayes S, Silvia WJ. Cellular mechanisms by which oxytocin stimulates uterine prostaglandin F2α synthesis in bovine endometrium: roles of phospholipases C and A2. Domest Anim Endocrinol. 1997;14(3):181-91. http://dx.doi.org/10.1016/S0739-7240(97)00003-9. PMid:9171976.

Carambula SF, Matikainen T, Lynch MP, Flavell RA, Gonçalves PB, Tilly JL, Rueda BR. Caspase-3 is a pivotal mediator of apoptosis during regression of ovarian corpus luteum. Endocrinology. 2002;143(4):1495-501. http://dx.doi.org/10.1210/endo.143.4.8726. PMid:11897708.

Fortier MA, Krishnaswamy K, Danyod G, Boucher-Kovalik S, Chapdalaine P. A postgenomic integratred view of prostaglandins in reproduction: implications for other body systems. J Physiol Pharmacol. 2008;59(1, Suppl 1):65-89. PMid:18802217.

Gijón MA, Leslie CC. Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. J Leukoc Biol. 1999;65(3):330-6. http://dx.doi.org/10.1002/jlb.65.3.330. PMid:10080535.

Ho KJ, Liao JK. Non-nuclear actions of estrogen: new targets for prevention and treatment cardiovascular disease. Mol Interv. 2002;2(4):219-28. http://dx.doi.org/10.1124/mi.2.4.219. PMid:14993393.

Hughes TL, Villa-Godoy A, Kesner JS, Fogwell RL. Destruction of bovine ovarian follicles: effects on the pulsatile release of luteinizing hormone and prostaglandin F2alpha-induced luteal regression. Biol Reprod. 1987;36(3):523-9. http://dx.doi.org/10.1095/biolreprod36.3.523. PMid:3474034.

Membrive CM, Cunha Pauline MD, Meirelles FV, Binelli M. Calcium potentiates the effect of estradiol on PGF2α production in the bovine endometrium. J Anim Sci Biotechnol. 2014;5(1):25. http://dx.doi.org/10.1186/2049-1891-5-25. PMid:24872882.

Thatcher WW, Terqui M, Thimonier J, Mauleon P. Effects of estradiol-17β on peripheral plasma concentration of 15-keto-13,14-dihydro PGF2α and luteolysis in cyclic cattle. Prostaglandins. 1986;31(4):745-56. http://dx.doi.org/10.1016/0090-6980(86)90178-4. PMid:3726154.

Razandi M, Pedram A, Greene GL, Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol Endocrinol. 1999;13(2):307-19. http://dx.doi.org/10.1210/mend.13.2.0239. PMid:9973260.

Oliveira ML. Ação do 17β-estradiol na síntese de PGF2α endometrial em vacas. [thesis]. Pirassununga: Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo; 2017. 106 f.

Danet-Desnoyers G, Meyer MD, Gross TS, Johnson JW, Thatcher WW. Regulation of endometrial prostaglandin synthesis during early pregnancy in cattle: effects of phospholipase and calcium in vitro. Prostaglandins. 1995;50(5-6):313-30. http://dx.doi.org/10.1016/0090-6980(95)00131-X. PMid:8838241.

Staggs KL, Austin KJ, Johnson GA, Teixeira MG, Talbott CT, Dooley VA, Hansen TR. Complex induction of bovine uterine proteins by interferon-τ. Biol Reprod. 1998;59(2):293-97. http://dx.doi.org/10.1095/biolreprod59.2.293. PMid:9687298.