STUDY ON LUTEOLYTIC MECHANISMS IN CATTLE: REGULATION OF ANTIOXIDANT ENZYMES BY PROSTAGLANDIN F2α AND REACTIVE OXYGEN SPECIES

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PREFACE

The experiments described in this dissertation were carried out at the Graduate School of Natural Science and Technology (JSPS Ronpaku [dissertation PhD] program), Okayama University, Japan, from October 2009 to July 2014, under the supervision of Associate Professor, Dr. Tomas J. ACOSTA (main supervisor), Professor, Dr. Kiyoshi OKUDA (co-supervisor) and Associate Professor DAM Van Tien (home supervisor).

This dissertation has not been submitted previously in whole or in part to a council, university or any other professional institution for a degree, diploma or other professional qualifications.

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ABSTRACT

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The corpus luteum (CL) forms in the ovary after ovulation and produces progesterone (P_4), which is essential for the establishment and maintenance of pregnancy. If pregnancy does not occur, the CL regresses from the ovary. Regression of the CL (luteolysis) is crucial to reset the ovarian cycle, so that the animal can return to estrus and have another opportunity to become pregnant. Prostaglandin F2α (PGF) is one of the main luteolytic factors in mammals. However, the mechanisms regulating the action and production of PGF in bovine CL remain unclear. Reactive oxygen species (ROS) is crucial for regulating the luteolytic action of PGF. The local concentration of ROS is controlled by antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Thus, antioxidant enzymes may be involved in regulating luteolysis in cow.

To clarify the roles of antioxidant enzymes in regulating the luteolytic action of PGF and ROS, we examined 1) the dynamic changes of antioxidant enzymes (SOD, CAT and GPx) in bovine CL at different stages of the estrous cycle and during luteolysis induced by PGF administration *in vivo* and 2) the dynamic relationship between PGF and ROS as well as its possible role in regulating antioxidant enzymes in bovine CL using cultured bovine luteal cells *in vitro*.

In chapter 2, corpora lutea were collected at the early (Days 2-3), developing (Days 5-6), mid (Days 8-12), late (Days 15-17) and regressing (Days 19-21) luteal stages (n = 5 CL/stage) and at 0, 2 and 24 h after luteolytic PGF administration (0 h) on Day 10 post ovulation (n = 5 cows/time point). Additional 5 CL were collected at the mid-luteal stage for immunohistochemical studies. During the estrous cycle, SOD1 protein expression was greater in the developing and mid-luteal stages than in the early, late and regressing-luteal stages ($P < 0.05$). Total SOD activity gradually increased from the early to mid-luteal stages, maintained a high level during the late-luteal stage and then decreased ($P < 0.05$) to the lowest level at the regressing-luteal stage. Catalase protein and the activities of CAT and GPx increased from the early to mid-luteal stage, and then decreased ($P < 0.05$), reaching their lowest levels at the regressing-luteal stage. The levels of GPx1 protein were lower in the regressing-luteal stage than in other stages

($P < 0.05$). Immunohistochemical examination also revealed the expression of CAT and GPx1 protein in bovine CL tissue. These findings provide evidence for a reduction in the antioxidant defenses against ROS during the regressing stage in bovine CL, and suggest that oxidative stress occurs during this stage to induce luteolysis. In addition, during PGF-induced luteolysis, injection of a luteolytic dose of PGF increased luteal SOD1 protein expression, total SOD activity, GPx1 protein expression and GPx activity at 2 h but suppressed them at 24 h. Catalase protein and CAT activity did not change at 2 h but CAT activity decreased ($P < 0.05$) at 24 h. These results indicate that during luteolysis PGF regulates bovine luteal antioxidant enzymes in a biphasic manner with an initial increase at 2 h followed by a decrease at 24 h. The down regulation of antioxidant enzymes during structural luteolysis may enhance ROS production and luteal cell death to ensure the regression of the bovine CL.

In chapter 3, luteal steroidogenic cells (LSCs) isolated from CL tissue ($n = 3$ CL per each experiment) at the mid-luteal stage (Days 8-12 of the estrous cycle) were treated with PGF and hydrogen peroxide (H_2O_2) for 2 h (mimicking functional luteolysis) or 24 h (mimicking structural luteolysis). Hydrogen peroxide stimulated PGF biosynthesis at 2 and 24 h in a dose- and time-dependent manner. Prostaglandin F2α, in turn, induced ROS production. Prostaglandin F2α (1 μM) and H_2O_2 (10 μM) increased SOD1 protein expression and total SOD activity, GPx1 protein and GPx activity at 2 h ($P < 0.05$) but suppressed them at 24 h ($P < 0.05$). Catalase protein expression and activity did not change at 2 h but they were suppressed at 24 h by PGF and H_2O_2 ($P < 0.05$). These findings confirmed that 1) LSCs are targets of the luteolytic action of PGF and 2) PGF, interacting with ROS, induced luteolysis by suppressing antioxidant enzymes in LSCs during structural luteolysis but not during functional luteolysis.

The overall results demonstrate that PGF through its interaction with ROS regulates the expressions and activities of the antioxidant enzymes SOD, CAT and GPx, in bovine CL, more specifically in LSCs, suggesting that these enzymes are involved in the mechanism of action of PGF in bovine CL. The down-regulation of these proteins and their activities during structural luteolysis could enhance the accumulation of reactive oxygen species, which would result in both increasing luteal PGF production and oxidative stress, to complete the CL regression in cattle.

CHAPTER 1
GENERAL MATERIALS AND METHODS

Chemicals

Analogue prostaglandin F2α (Dinoprost, Dinolytic) was purchased from Pharmacia & Upjohn, Belgium. The culture medium (Dulbecco Modified Eagle Medium [DMEM] & Ham's F-12 [1:1 [w/w]], no. D8900) and glycerol (no. G7757) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Calf serum (CS, no. 16170–078) and gentamicin (no. 15750-060) were purchased from Life Technologies (Grand Island, NY, USA). CellROX™ Deep Red Reagent (fluorogenic probe for ROS detection), NucBlue™ Live Cell Stain (for cellular nucleus detection, Hoechst 33342) and nitrocellulose membrane for Western blot (LC2000) were purchased from Invitrogen. SOD1 primary antibody (goat SOD1 polyclonal antibody, no. sc-8637) and secondary antibody for SOD (anti-goat Ig, HRP-linked whole antibody produced in donkey, sc-2020) were purchased from Santa Cruz (Santa Cruz, CA, USA). Catalase (CAT) primary antibody (Anti-Catalase [Bovine liver] Rabbit, no. 200-4151) was purchased from Rockland Immunochemicas Inc. (Gilbertsville, PA, USA); Glutathione peroxidase 1 (GPx1) primary antibody (Rabbit polyclonal antibody, anti-Glutathione peroxidase 1, no. ab22604) was purchased from Abcam (Cambridge, USA). Secondary antibody for catalase and GPx1 (anti-rabbit Ig, HRP-linked whole antibody produced donkey, no. NA934) was purchased from Amersham Biosciences Corp. (San Francisco, CA, USA). Beta actin primary antibody (mouse ACTB monoclonal antibody (no.A2228) was purchased from Sigma-Aldrich. Beta actin secondary antibody (anti-mouse Ig, HRP-linked whole antibody produced in sheep, no. NA931) and ECL Western blotting detection system (RPN2109) were purchased from Amersham Biosciences (Buckinghamshire, UK). SOD assay kit - WST (S311-08) was purchased from DOJINDO (Kumamoto, Japan). Complete Protease Inhibitor (no. 11 697 498 001) was purchased from Roche Diagnostics (Basel, Switzerland). Catalase Activity Assay Kits (no. K773-100) were purchased from BioVision (Mountain View, CA94043, USA). GPx Assay Kits (no. 703102) were purchased from Cayman (Ann Arbor, Michigan 48108, USA). Secondary antibody for immunohistochemical trial of CAT and GPx protein expression (ImmPRESS™ Universal Reagent Kit, no. MP-7500) was purchased from Vector Laboratories (Burlingame, CA, USA). Peroxidase substrate (DAB-buffer tablets) was purchased from Merck KGaA (Darmstadt, Germany).

Animal tissue collection

Collection of bovine corpus luteum tissues throughout the luteal stages

Uteri and ovaries with CL were collected from Holstein cows at a local slaughterhouse within 10-20 min after exsanguinations and transported to the laboratory (Laboratory of Reproductive Physiology, Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan) within 1-1.5 h on ice. Only ovaries containing CL from apparently normal reproductive tracts based on uterine characteristics (size, color, tonus, consistency and mucus) were used in the present study. Luteal stages were classified as early (Days 2–3 after ovulation), developing (Days 5–7), mid (Days 8–12), late (Days 15–17) and regressed (Days 19–21) luteal stages by macroscopic observation of the ovary and corpus luteum as described previously [1-3]. The CL tissues were immediately used for cell isolation and cell culture (CL tissue at mid luteal stage, n = 3 CL per each experiment), fixed for immunohistochemical trial (CL tissue at mid luteal stage, n = 5 CL), or dissected from the ovaries and stored at -80°C (n = 5 CL per each luteal stage) until the protein and enzymes activity analyses.

Collection of bovine corpus luteum tissues during prostaglandin F2α (PGF)-induced luteolysis

The collection procedures were approved by the local institutional animal care and use committee of the Polish Academy of Sciences in Olsztyn, Poland (Agreement No. 5/2007, 6/2007 and 88/2007). Healthy, normally cycling Polish Holstein Black and White cows were used for collection of CL. Estrus was synchronized in the cows by two injections of a PGF analogue (PGFa, Dinoprost, Dinolytic; Pharmacia & Upjohn, Belgium) with an 11-day interval according to the manufacturer's direction. Ovulation was determined by a veterinarian via transrectal ultrasonograph examination. Then, corpora lutea were collected by the Colpotomy technique using a Hauptner's effeninator (Hauptner and Herberholz, Solingen, Germany) on Day 10 post ovulation, i.e., just before administration of a luteolytic dose of a PGF analogue (PGFa; 0 h) and at 2 and 24 h post-treatment (n = 5 cows per time point). CL tissues were dissected from the ovaries and then immediately stored at -80°C until the protein expression and enzyme activity analysis.

Cell isolation

CL of Holstein cows were collected from a local slaughterhouse as described in the section of collection of bovine CL tissues at mid-luteal stage (Days 8-12). Luteal cells were obtained as described previously [4]. Briefly, bovine CL tissues at mid-luteal stage (n = 3 CL per each experiment) were enzymatically dissociated and the resulting cell suspensions were centrifuged (5 min at 50 x g) three times to separate the luteal cells (pellet) from other types of luteal nonsteroidogenic cells. The dissociated luteal cells were suspended in a culture medium (Dulbecco modified Eagle medium and Ham F-12 medium (1:1 [v/v]; no. D8900; Sigma-Aldrich Inc., St. Louis, MO, USA) containing 5% calf serum (no. 16170–078; Life Technologies Inc., Grand Island, NY, USA) and 20 µg/ mL gentamicin (no. 15750–060; Life Technologies Inc.). Cell viability was greater than 90%, as assessed by trypan blue exclusion. The cells in the cell suspension after centrifugation consisted of about 70% small and 20% large luteal steroidogenic cells (LSCs), 10% endothelial cells or fibrocytes, and no erythrocytes.

Cell culture

The dispersed luteal cells were seeded at 2 x 10^5 viable cells per 1 mL in 24-well cluster dishes (no. 662160; Greiner Bio-One) for examining the PGF production; or in 6 mL culture flasks (no. 658175; Greiner Bio-One) for determining SOD1, CAT and GPx1 protein expression or SOD, CAT and GPx activity. Luteal cells were also cultured in 6-well plates containing collagen coated coverslips for determining intracellular ROS production. Cells were cultured in a humidified atmosphere with 5% CO_2 in air at 38°C in an N_2- O_2- CO_2- regulated incubator (no. BNP-110; ESPEC CORP.). After 12 h of culture, the medium was replaced with fresh medium containing 0.1% BSA, 5 ng/mL sodium selenite and 5 µg/mL transferrin, and then treated with PGF (0.1, 1 or 10 µM) or H_2O_2 (1, 10 or 100 µM). The doses of PGF and H_2O_2 were determined in our preliminary experiments to confirm that these doses do not affect the viability of the cultured cells [3]. After 2 h (mimicking functional luteolysis) or 24 h (mimicking structural luteolysis) of incubation, the cultured cells and/or media were collected and stored at -80°C until further analysis.

Superoxide dismutase-1 protein expression

Superoxide dismutase (SOD)-1 protein expression in luteal tissue and in cultured luteal cells was assessed by Western immunoblotting analysis. Tissues or cells were lysed in 150 µL lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100 [Bio-Rad Laboratories], 10% glycerol [G7757; Sigma-Aldrich], Complete [11 697 498

001; Roche Diagnostics, Basel, Switzerland], pH 7.4). Protein concentrations in the lysates were determined by the method of Osnes et al. [5], using BSA as a standard. The proteins were then solubilized in SDS gel-loading (10% glycerol, 1% β-mercaptoethanol [137–06862; Wako Pure Chemical Industries, Ltd.], pH 6.8) and heated at 95°C for 10 min. Samples (50 μg protein) were electrophoresed on a 15% SDS-PAGE for 1.5 h at 30 mA.

The separated proteins were electrophoretically transblotted to a 0.2-μM nitrocellulose membrane (LC2000; Invitrogen) at 300 mA V for 3 h in transfer buffer (25 mM Tris–HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris–HCl, 137 mM NaCl, pH 7.5]), incubated in blocking buffer (5% nonfat dry milk in TBS-T) for 1 h at room temperature, incubated at 4°C with a primary antibody specific to each protein (goat SOD1 polyclonal antibody [23 kDa; 1:500 in TBS-T, overnight; sc-8637; Santa Cruz Biotechnology, Santa Cruz, CA, USA] and mouse ACTB monoclonal antibody [internal standard, 42 kDa; 1:4000 in TBS-T, overnight; A2228; Sigma-Aldrich]).

The membrane was washed three times for 5 min in TBS-T at room temperature, incubated with secondary antibody (SOD1 [1:10,000 in TBS-T]: anti-goat Ig, HRP-linked whole antibody produced in donkey, sc-2020; Santa Cruz; ACTB [1:40,000 in TBS-T]: anti-mouse Ig, HRP-linked whole antibody produced in sheep, NA931; Amersham Biosciences, Buckinghamshire, UK) for 1 h, and washed three times in TBS for 5 min at room temperature. The signal was detected by an ECL Western immunoblotting detection system (RPN2109; Amersham Biosciences).

The intensity of the immunological reaction was estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health; Bethesda, MD, USA).

Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity in luteal tissues or in cultured luteal cells at the end of the incubation period was determined by using a SOD assay kit - WST (S311-08; DOJINDO laboratories, Kumamoto, Japan). Superoxide dismutase (SOD) activity was calculated according to the manufacturer's direction and expressed as inhibition rate. The principle of total SOD activity assay was based on the inhibition of WST-1 reduction. Superoxide anions are generated from the conversion of xanthine and O_2 to uric acid and H_2O_2 by xanthine oxidase (XOD). The superoxide anion then converts a water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) into a water-soluble formazan dye, a colored product that absorbs light. Addition of SOD to this reaction reduces superoxide ion levels, thereby lowering the rate of water-soluble formazan dye formation. Total SOD activity in the experimental sample was measured as the percent inhibition of the rate of formazan dye formation. One unit of SOD is the amount of enzyme in 20 μL of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%.

Catalase and glutathione peroxidase-1 protein expression

Protein expressions for catalase (CAT) and glutathione peroxidase-1 (GPx1) in CL tissue and cultured luteal cells were assessed by Western blotting analysis. Tissue or cells were lysed in 150 μL homogenizing buffer (20 mM Tris-HCl, 150 nM NaCl, 1% Triton X-100 [Bio-Rad Laboratories], 10% glycerol [G7757; Sigma-Aldrich], Complete Protease Inhibitor [11 697 498 001; Roche Diagnostics, Basel, Switzerland], pH 7.4). Protein concentrations in the homogenizing buffer were determined by the method of Osnes et al. [5], using BSA as a standard. The proteins were then solubilized in SDS gel-loading buffer (10% glycerol, 1% β-mercaptoethanol [137-068662; Wako Pure Chemical Industries, Ltd.], pH 6.8) and heated at 95°C for 10 min. Samples (50 μg protein) were electrophoresed on a 15% SDS-PAGE for 90 min at 200 V, 250 mA. The separated proteins were electrophoretically transblotted to a 0.2 μM nitrocellulose membrane (LC2000; Invitrogen) at 200 V, 250 mA for 3 h in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3).

The membrane was washed in TBS (25 mM Tris-HCl, 137 mM NaCl, pH 7.5), incubated with blocking buffer (5% nonfat dry milk in TBS-T [0.1% Tween 20 in TBS]) for 1 h at room temperature, and washed in TBS-T [25 mM Tris-HCl, 137 mM NaCl, pH 7.5]. The membranes were then incubated separately with a primary antibody in blocking buffer specific to each protein: 1) Anti-Catalase [Bovine liver] Rabbit [60 kDa; 1:10,000; no. 200-4151; Rockland Immunochemicas Inc., Gilbertsville, PA, USA]; 2) Rabbit polyclonal antibody, anti-Glutathione peroxidase 1 [22 kDa, 1 μg/mL; no. ab22604; Abcam, Cambridge, USA]; 3) Mouse beta-actin antibody [42 kDa; 1: 4000; no. A2228; Sigma-Aldrich].

After primary antibody incubation for overnight at 4°C, the membranes were washed for 5 min, five times in TBS-T at room temperature, incubated with blocking buffer for 10 min. The membranes were then incubated for 1 h with secondary polyclonal antibody: 1) Anti-rabbit Ig, HRP-linked whole antibody produced donkey [Amersham Biosciences Corp.; San Francisco, CA, USA; no. NA934] for CAT [1:10,000] and GPx [1:4000]; 2) Anti-mouse, HRP-linked whole antibody produced in sheep [Amersham Biosciences Corp.; no. NA931] for beta-actin [ACTB; 1:40,000]. Then, the membranes were washed for 10 min, two times in TBS-T at room temperature. After that, protein bands were developed by the Enhanced ChemiLuminescence (ECL) Western blotting detection system (RPN2109; Amersham Biosciences) or by Molecular Imager ® Gel Doc™XR+ and ChemiDoc™XRS+ Systems using Image Lab software 4.0.1 (Biorad).

Finally, protein band in the images obtained from scanned radiographic film or from the Molecular Imager were quantified using ImageJ software (Windows version of NIH Image, http://rsb.info.nih.gov/nih-image/, National Institutes of Health). Relative density was quantified by normalization of the integrated density of each blot to that of the corresponding ACTB.

Catalase activity assay

Catalase (CAT) activity in CL tissue or in cultured cells at the end of incubation period was determined using a commercially-available Catalase Activity Assay Kit (BioVision, No. K773-100, Mountain View, CA94043, USA). In the assay, catalase first reacts with H_2O_2 to produce water and oxygen. The unconverted H_2O_2 reacts with OxiRed™ probe to produce a product, which can be measured by a colorimetric method. Briefly, tissue or cells homogenized in cold assay buffer were centrifuged at 10,000×g for 15 min at 4°C and the supernatants were collected for the assay. The assay was performed in triplicate using 96-well microplates. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 570 nm using an absorbance microplate reader (Model 680, Bio-Rad Laboratories, Inc. 1000 Alfred Nobel Dr. Hercules, CA, 94547 USA). One unit of CAT was defined as the amount of enzyme needed to decompose 1 μM of H_2O_2 in 1 min. The activity of CAT was normalized to milligram of protein used in the assay and was expressed as mU/mg protein.

Glutathione peroxidase activity assay

Glutathione peroxidase (GPx) activity in CL tissue or in cultured cells at the end of incubation period was determined using GPx Assay Kit (Cayman, No. 703102, Ann Arbor, Michigan 48108, USA) based on the change in absorbance at 340 nm (Δ340 nm/min) as it is described in the user's manual included in the kit. Results are presented as micro mol/min/mg protein. Principally, GPx protect the cell from oxidative damage catalyzing the reduction of hydroperoxides, including H_2O_2, by reduced glutathione. With the exception of phospholipid-hydroperoxide GPx, a monomer, all

GPx enzymes are tetramers of four identical subunits. Each subunit contains a selenocysteine in the active site, which participates directly in the two-electron reduction of the peroxide substrate. The enzyme uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine. The Cayman Chemical Glutathione Peroxidase Assay Kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. Oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A340 is directly proportional to the GPx activity in the sample [6]. Glutathione peroxidase activity was expressed as micromoles of NADPH oxidized. The results were normalized to milligram of protein used in the assay.

Statistical analysis

Data of SOD1, CAT and GPx1 protein level, and SOD, CAT and GPx activity were obtained from five separate experiments, each performed in triplicate. Luteal tissues were collected from different cows at different luteal stages (n = 5/stage) and at different time points post-PGF injection (n = 5 cows/time point). The statistical significance of differences in the amounts of SOD1, CAT and GPx1 protein, SOD, CAT and GPx activity, PGF and ROS production were analyzed using one-way ANOVA followed by Fisher’s protected least-significant difference (PLSD) procedure as multiple comparison tests. Data were expressed as the mean ± SEM. Means were considered significant difference when P value is less than 0.05.

CHAPTER 2

CHANGE IN ANTIOXIDANT ENZYMES IN THE BOVINE CORPUS LUTEUM THROUGHOUT THE ESTROUS CYCLE AND DURING PROSTAGLANDIN F2α –INDUCED LUTEOLYSIS *IN VIVO*

Introduction

The corpus luteum (CL) forms in the ovary after ovulation and produces progesterone (P_4), the hormone responsible for the maintenance of pregnancy [7]. If pregnancy does not occur, the CL regresses and loses its capacity to produce P_4 [8, 9]. Regression of the CL (luteolysis) is crucial to reset the ovarian cycle, so that the animal can return to estrus and have another opportunity to become pregnant [10].

Prostaglandin F2α (PGF) is well-known as a luteolytic factor in mammals. In the cow, both endogenous PGF synthesized by the uterus at the late-luteal stage [9] and exogenous PGF given during the mid-luteal stage [11] cause irreversible luteal regression that is characterized by a rapid decrease in P_4 production (functional luteolysis) followed by a decrease in the size of the CL (structural luteolysis) [12, 13]. In addition, the CL is reported to be able to synthesize PGF in the cow [14] and ewe [15, 16]. Luteal PGF is proposed to induce luteolysis via a paracrine and/or autocrine mechanism [17]. However, the mechanisms regulating the luteolytic action of PGF remain unclear.

Reactive oxygen species (ROS), the byproducts of normal aerobic metabolism, are highly cytotoxic, and thus act as apoptotic factors [18]. ROS include superoxide radicals, hydrogen peroxide and hydroxyl radicals [19]. The cellular concentration of ROS is controlled by antioxidant enzymes. The balance between ROS generation and ROS elimination by antioxidant enzymes helps to maintain cellular function, i.e., an increase in ROS production or a decrease in antioxidant enzyme levels or activities leads to an overall increase in intracellular ROS levels and causes cell death [18]. ROS have been implicated in the regulation of luteal function, including luteolysis [20, 21]. ROS generation is induced by PGF in the ovine [22] and rat [23] CL. PGF production in turn is induced by ROS in human decidua [24]. However, the mechanisms underlying the interaction between PGF and ROS in the bovine corpus luteum are unclear.

Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx). SOD protect the cells from superoxide radical (O_2^-).

Under the action of SOD, O_2^- is transformed into hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) [25]. Moreover, because of its ability to scavenge O_2^-, SOD protect cells against the single oxygen (O) and OH^-, the products of the reaction between O_2^- and H_2O_2, which are even more reactive and cytotoxic than either O_2^- or H_2O_2 [18, 26]. In mammalian tissues, three types of SOD have been identified. SOD1 is located in the cytosol and nucleus, SOD2 is present in the mitochondria and SOD3 is located in the extra-cellular matrix of tissues [27]. SOD1 is widely distributed and comprises 90% of the total SOD activity [28]. By contrast, catalase is usually located in a cellular organelle called the peroxisome [29]. Glutathione peroxidases include several isozymes that differ according to their cellular location and substrate specificity [30]. Glutathione peroxidase type 1 (GPx1), the most abundant type of GPx located in the cytoplasm [30]. Both CAT [31] and GPx [32] protect the cells by conversion of SOD produced- H_2O_2 into water and oxygen [32]. All of these antioxidant enzymes are found in nearly all living organisms exposed to oxygen [31]. Since the local concentrations of ROS are controlled by antioxidant enzymes, it is possible that these enzymes are involved in regulating the luteolytic action of PGF [33].

In the present study, we examined the dynamic changes of SOD, CAT and GPx, in bovine CL at different stages of the estrous cycle and during PGF-induced luteolysis. The cellular localization of CAT and GPx1 in the luteal tissue were also examined.

Materials and methods

Localization of catalase (CAT) and glutathione peroxidase-1 (GPx1) protein by immunohistochemistry.

Bovine corpus luteum tissues at mid-luteal stage (Days 8-12, n = 5 CL) were used for immunohistochemical trials. Whole CL were fixed overnight in 10% phosphate buffer (PBS) formalin and prepared for immunohistochemistry. Briefly, the tissue was processed for paraffin embedding. Six micron tissue sections were cut from paraffin-embedded blocks and processed for immunohistochemistry using the ImmPRESS™ Universal Reagent Kit (No. MP-7500, Vector Laboratories, Burlingame, CA, USA). Slides were rinsed extensively in PBS, treated with diluted normal horse blocking serum followed by 1 hour incubation with primary antibody of CAT (Anti-Catalase [Bovine liver] Rabbit [1:300 dilution; no. 200-4151; Rockland Immunochemicas Inc., Gilbertsville, PA, USA]) or GPx1 (Rabbit polyclonal antibody, anti-Glutathione peroxidase 1 [1:300 dilution; no. ab22604; Abcam]), respectively. Following incubation at room temperature, sections were washed in PBS, incubated with immPRESS^{TM} reagent (Vector Laboratories) and washed in PBS. Then sections were incubated in peroxidase substrate solution (DAB-buffer tablets, Merck KGaA, Darmstadt, Germany) and counterstained with Mayer's Hematoxylin. Tissue processed in the same manner, without CAT or GPx1 primary antibody were used as negative immunoreactivity. The sections were washed in distilled water, dehydrated in a graded series of ethanol, and cleared in xylene, coverslipped and observed under light field microscope. For the examination of the expression of CAT or GPx1 protein in the luteal cells, 3 cross-sections (slide) per CL were randomly selected. In each slide, 3 microscope fields were randomly selected for examination. Brown color detected in the cytoplasm of the luteal cells indicated the presence of CAT or GPx1 protein.

Results

Localization of catalase (CAT) and glutathione peroxidase-1 (GPx1) protein by immunohistochemistry

Immunohistochemical examination revealed the expression (brown color) of CAT (Fig.1B, C) and GPx1 (Fig. 2B, C) protein in bovine mid-luteal stage CL tissue, more specifically in large luteal steroidogenic cells (LSCs), small LSCs as well as luteal endothelial cells (LECs).

Dynamic changes in antioxidant enzymes protein expression and their activities in bovine corpus luteum throughout the luteal stages

The level of SOD1 protein was greater in the developing and mid-luteal stages than in the early, late and regressing-luteal stages ($P < 0.05$; Fig. 3A). Total SOD activity (Fig. 3B) gradually increased from the early to mid-luteal stages, maintained a high level during the late-luteal stage and then decreased ($P < 0.05$) to the lowest level at the regressing-luteal stage.

CAT protein expression (Fig. 4A) and the activity (Fig. 5A) and GPx activity (Fig. 5B) increased from the early to mid-luteal stage, then all decreased ($P < 0.05$), reaching their lowest levels at the regressing luteal stage. The GPx1 protein expression gradually decreased from the developing to the regressing-luteal stage (Fig. 4B). The GPx1 protein expression level was significantly lower at the regressing luteal stage than at other stages ($P < 0.05$) ($n = 5$ CL per stage).

Dynamic changes in antioxidant enzymes protein expression and their activities in bovine corpus luteum during prostaglandin F2α (PGF)-induced luteolysis in vivo

Following administration of a luteolytic dose of a PGF analogue (0 h), the expression of SOD1 protein (Fig. 6A) as well as total SOD activity (Fig. 6B) in CL tissues biphasically changed with an initial increase at 2 h followed by a decrease at 24 h post-treatment ($P < 0.05$).

An injection of a luteolytic dose of PGF significantly increased luteal GPx1 protein expression (Fig. 7B) and GPx activities (Fig. 8B) at 2 h but suppressed it at 24 h. Catalase protein expression (Fig. 7A) and CAT activity (Fig. 8A) did not change at 2 h but CAT activity significantly decreased ($P < 0.05$) at 24 h.

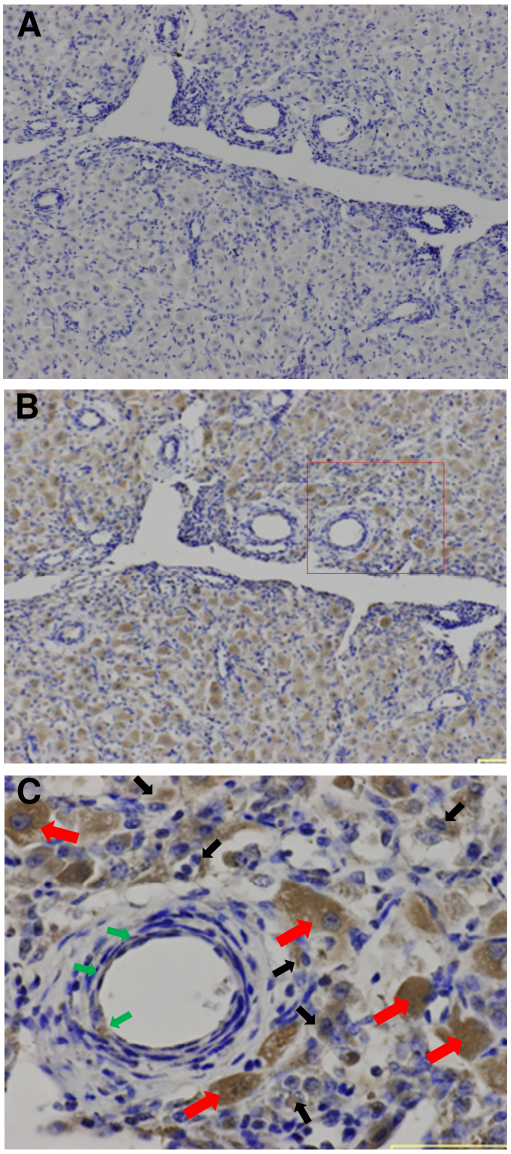

Figure 1. Representative images of immunohistochemical expression of catalase (CAT) protein in corpora lutea from cycling cow.

Images A and B showed sections of luteal tissue with negative and positive CAT expression (scale bar = 50 μm), respectively. Image C was a part of image B at higher magnification (scale bar = 50 μm). The arrows showed examples of large luteal steroidogenic cells (LSCs) (red arrows), small LSCs (black arrows) as well as luteal endothelial cells (LECs) (green arrows) expressing the CAT protein

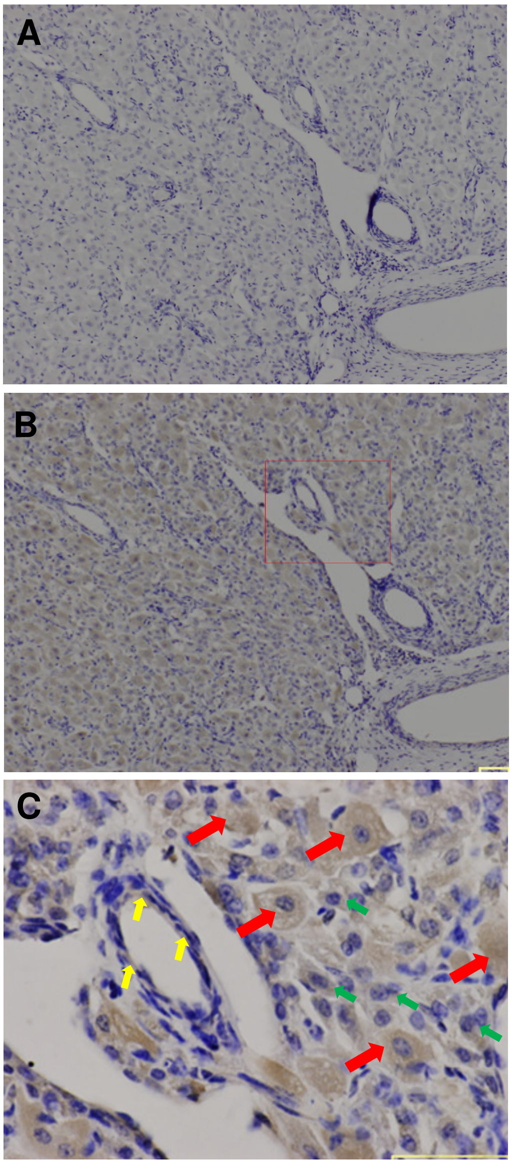

Figure 2. Representative images of immunohistochemical expression of glutathione peroxidase-1 (GPx1) protein in corpora lutea from cycling cow.

Immunohistochemical representative pictures of GPx1 were shown. Picture A was negative control while picture B was positive staining (scale bar = 50 µm). Image C was a part of image B at higher magnification (scale bar = 50 µm). The arrows showed examples of large LSCs (red arrows), small LSCs (green arrows) and LECs (yellow arrows) expressing the GPx1 protein.

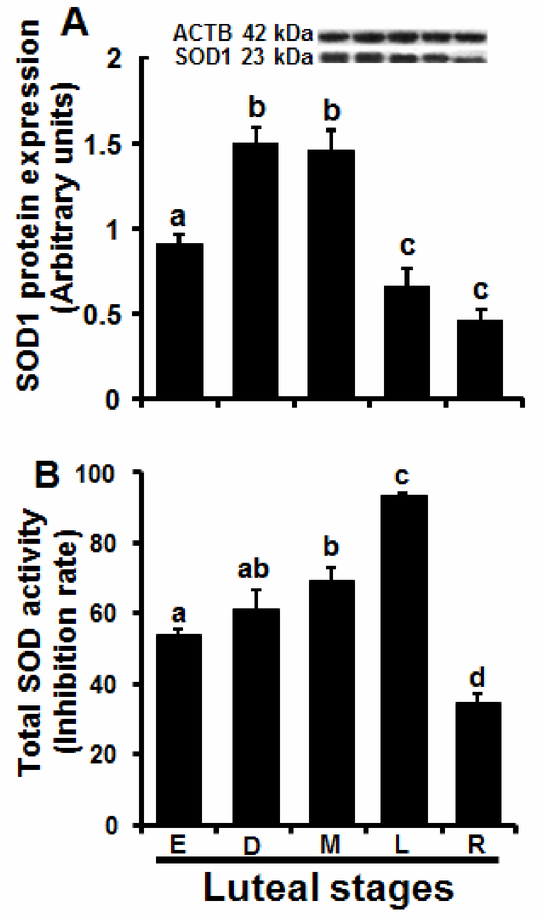

Figure 3. Changes in superoxide dismutase (SOD)-1 protein expression and total SOD activity in bovine corpus luteum throughout the luteal stages

Changes in relative amounts of SOD1 protein expression (Fig. 3A) and total SOD activity (Fig. 3B) in bovine CL throughout the luteal stages (early [E], Days 2-3; developing [D], Days 5-6; mid [M], Days 8-12; late [L], Days 15-17; regressing [R], Days 19-21). Data are the mean ± SEM for five samples per stage. Representative samples of Western blot for SOD1 and ACTB are shown in the upper panel of B, respectively. Total SOD activity was determined by a colorimetric method using an SOD assay kit-WST as described in the chapter 1 (General materials and methods). Different superscript letters indicate significant differences ($P < 0.05$) between luteal stages as determined by ANOVA followed by protected least significant difference test.

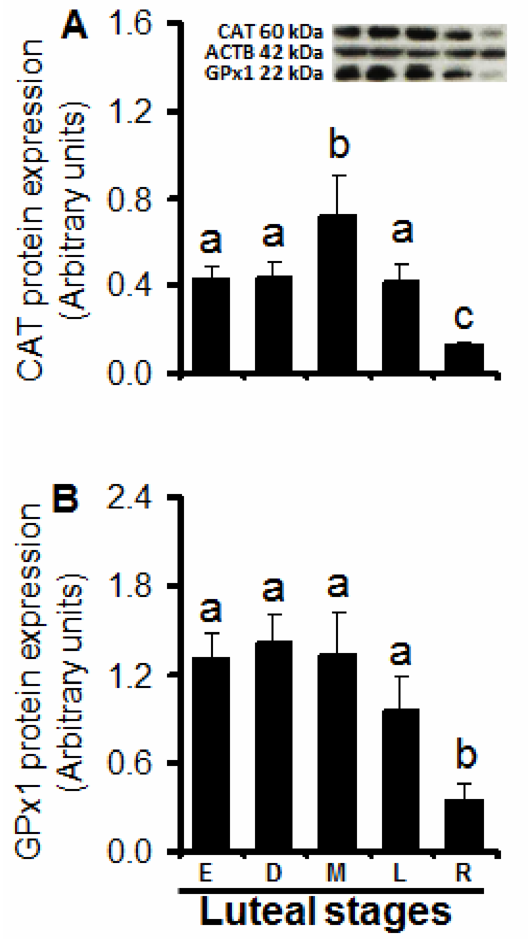

Figure 4. Changes in catalase and glutathione peroxidase-1 protein expression in luteal tissue throughout the estrous cycle.

Changes in catalase (CAT) and glutathione peroxide 1 (GPx1) protein expression in luteal tissue throughout the luteal stages (early [E], Days 2-3; developing [D], Days 5-6; mid [M], Days 8-12; late [L], Days 15-17; regressing [R], Days 19-21). Data are the mean ± SEM for five samples per luteal stage. Catalase protein expression (A), GPx1 protein expressions (B) were assessed by Western blotting. Representative samples of Western blot for CAT, GPx1 and ACTB (internal control) are shown in the upper panel of Fig. 4A. Different superscript letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by protected least significant difference test.

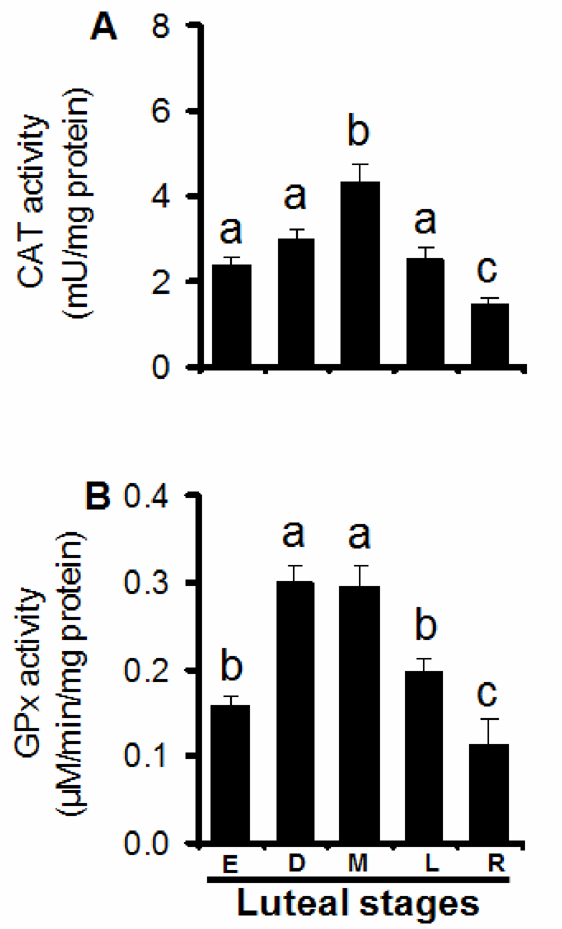

Figure 5. Changes in catalase and glutathione peroxidase activity in luteal tissue throughout the estrous cycle.

Changes in catalase (CAT) and glutathione peroxide (GPx) activity in luteal tissue throughout the luteal stages (early [E], Days 2-3; developing [D], Days 5-6; mid [M], Days 8-12; late [L], Days 15-17; regressing [R], Days 19-21). Data are the mean ± SEM for five samples per luteal stage. The enzyme activity of CAT (Fig. 5A) and GPx (Fig. 5B) were determined by colorimetric method using commercial assay kit (CAT assay kit, Bio Vision and GPx assay kit, Cayman), respectively. Data are the mean ± SEM (n = 5 samples per luteal stage). Different superscript letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by protected least significant difference test.

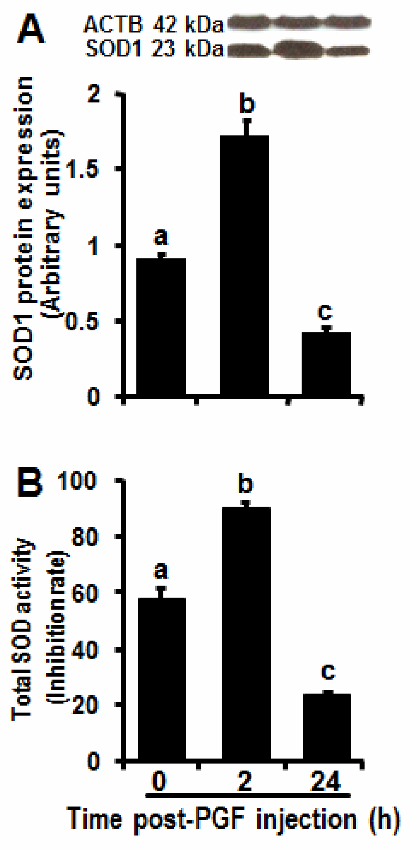

Figure 6. Change in superoxide dismutase (SOD)-1 protein expression and total SOD activity in bovine corpus luteum during prostaglandin F2α (PGF)-induced luteolysis.

Bovine CL tissue collected just before (0 h, control) and after administration (2 h, 24 h) of luteolytic dose of PGF. Protein expression of SOD1 (Fig. 6A) was assessed by Western blot. Representative samples of Western blot for SOD1 and ACTB (internal control) are shown in the upper panel of Fig. 6A, respectively. Total SOD activity was determined by a colorimetric method using an SOD assay kit-WST. Data are the mean ± SEM (n = 5 samples per time point). Different superscript letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by protected least significant difference test.

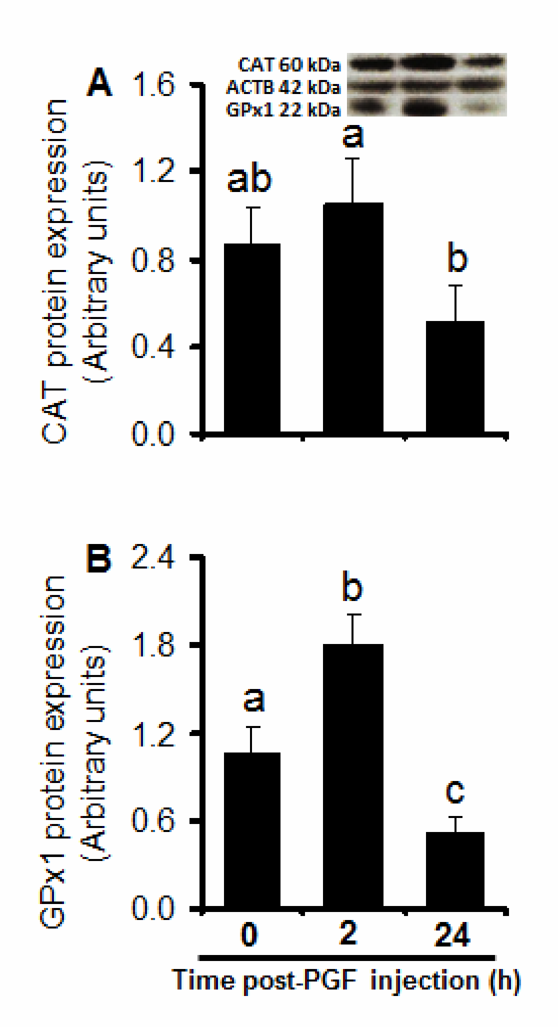

Figure 7. Changes in catalase (CAT) and glutathione peroxidase-1 (GPx1) protein expression in luteal tissue during prostaglandin F2α (PGF)-induced luteolysis.

Bovine CL tissue collected just before (0 h, control) and after administration (2 h, 24 h) of luteolytic dose of PGF. Protein expressions of CAT (Fig. 7A) and GPx1 (Fig. 7B) were assessed by Western blot. Representative samples of Western blot for CAT, GPx1 and ACTB (internal control) are shown in the upper panel of Fig. 7A. Data are the mean ± SEM (n = 5 samples per time point). Different superscript letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by protected least significant difference test.

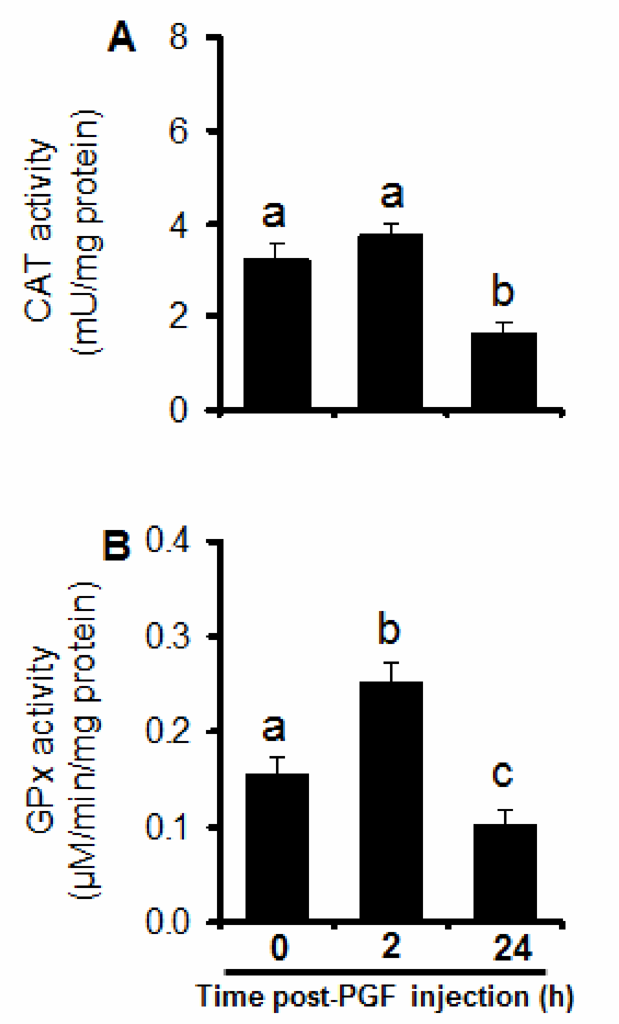

Figure 8. Changes in catalase (CAT) and glutathione peroxidase (GPx) activity in luteal tissue during prostaglandin F2α (PGF)-induced luteolysis.

Bovine CL tissue collected just before (0 h, control) and after administration (2 h, 24 h) of luteolytic dose of PGF. The enzyme activity of CAT (Fig. 8A) and GPx (Fig. 8B) were determined by colorimetric method using commercial assay kit (CAT assay kit, Bio Vision and GPx assay kit, Cayman), respectively. Data are the mean ± SEM (n = 5 samples per time point). Different superscript letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by protected least significant difference test.

Discussion

The present study demonstrated that antioxidant enzymes are expressed in bovine luteal tissues. The protein expression and activity of SOD, CAT and GPx were down-regulated in the regressing luteal stage of the estrous cycle as well as during structural luteolysis induced by PGF *in vivo*. These results provide evidence for a reduction in the defenses against ROS during structural luteolysis in cow, and suggest that oxidative stress occurs during luteolysis, leading to luteal cell death and luteolysis.

In cows, regression of the CL is induced by the episodic pulsatile secretion of uterine PGF starting between Days 17 and 19 of the estrous cycle [9]. Previous studies have reported that PGF increases the production of ROS rats [23, 34]. ROS have been demonstrated to stimulate PGF production [35, 36]. Since antioxidant enzymes are ROS scavengers, the investigation of the mechanism controlling luteal antioxidant enzymes is crucial to understanding the luteolytic cascade induced by PGF. In the present study, immunohistochemical examination revealed the expression of CAT and GPx1 proteins in bovine CL tissue, more specifically in large luteal steroidogenic cells (LSCs), small LSCs as well as luteal endothelial cells (LECs). These preliminary results provide evidence for the presence of antioxidant enzymes in the bovine CL. In addition, we found that the protein expression and activity SOD, CAT and GPx are higher in the early to late-luteal stage than in the regressing-luteal stage, suggesting that the balance between antioxidant enzymes and ROS in the bovine CL at early to late-luteal stage leans to antioxidant enzymes. In other words, antioxidant enzymes may help the cells to overcome the detrimental effect of ROS and that the CL keeps its structures and/or functions during these stages. The changes in protein expression and activity of CAT during the estrous cycle observed in the present study agree with the earlier findings of Rueda et al. [37] in which CAT mRNA was significantly (154%) higher in functional CL than in the regressed CL. Our results are also in accordance with those of earlier observations of Nakamura et al. [38], in which CAT was highly expressed at the middle stages of the estrous cycle.

By contrast, during the regressing-luteal stage in which PGF has a luteolytic effect [39], all of SOD1 protein expression, total SOD activity, CAT and GPx protein expression and activity decreased to the lowest level. In rats, the level of luteal Cu/Zn-SOD decreased and remained at low levels during luteal regression [40]. In the human CL, Cu/Zn-SOD activity was the lowest during the regression phase [41]. Rueda et al. [37] reported a decline of Manganese-containing SOD in the regressed bovine CL.

Rapoport et al. [42] found that CAT activity decreased concomitantly with the decrease in P_4 during the regressing stage of bovine estrous cycle. In addition, Nakamura et al. [38] found that GPx levels gradually decrease as the estrous cycle progresses and that H_2O_2 produced due to the lack of GPx is a potent inducer of luteal cell apoptosis. These findings strongly support the concept that PGF induces luteal regression by suppressing the protective role of antioxidant enzymes in the bovine corpus luteum.

Since 1960, estrous synchronization in cattle was recognized as an important procedure for artificial insemination (AI) [43]. From that time, PGF analogue has been widely studied and used for estrous synchronization. Exogenous PGF given during the mid-luteal stage of the bovine CL [11] induces irreversible luteolysis. Despite intensive investigation, the mechanisms by which PGF causes luteal regression remain undetermined. Several studies have been focused on the possible role of reactive oxygen species (ROS) in mediating the life span of the corpus luteum [8, 10, 19] and evidences for the concept that ROS interacts with PGF to induce luteolysis are also being accumulated [22]. We recently observed that an injection of PGF induces a transient (1–2 h) increase in the partial pressure of oxygen (pO_2) in ovarian venous blood [44], and that the pO_2 of venous blood is higher in the ovarian vein than in the jugular vein in cow suggesting that luteal microenvironment seems to be exposed to high O_2 condition (hyperoxia), especially during the short period of time (1–2 h) following PGF treatment. Hyperoxia condition can be toxic for the cells due to excessive production and accumulation of ROS [45]. Moreover, the rat CL produces significant amounts of ROS [34] and increases ROS (H_2O_2) generating capacity within a few hours after injection of a luteolytic dose of PGF [23, 46]. Taken together, ROS seem to be involved in the luteolytic cascade induced by PGF during the surge secretion of PGF from endometrium and during exogenous PGF administration in cattle. The increase in ROS generation could be due to the down-regulation of ROS scavenging systems (antioxidant enzymes). In the present study, following administration of a luteolytic dose of a PGF analogue, the expression of SOD1 protein as well as total SOD activity in CL tissues was decreased at 24 h post-treatment. An injection of a luteolytic dose of PGF significantly suppressed luteal GPx1 protein expression, CAT activity GPx activities at 24 h. These finding again support for the concept that down regulation of antioxidant enzymes during structural luteolysis may enhance ROS production and luteal cell demise to ensure the regression of the bovine CL.

Surprisingly, in the present study, injection of a luteolytic dose of PGF increased luteal SOD1 protein expression, total SOD activity, GPx1 protein expression and GPx activity at 2 h. These findings were unexpected and suggest that PGF only suppresses the protective role of antioxidant enzymes during structural luteal regression but not during functional luteal regression. The reason for the increase in the antioxidant defences against ROS during functional luteolysis *in vivo* might be due to the activation of the neuro-endocrine stress axis.

The overall results provide evidence for the protective role of antioxidant enzyme in maintaining CL function during early to late luteal stage in bovine CL. A decrease in these antioxidant enzymes proteins and their activities during regressing luteal stage as well as during structural luteolysis induced by PGF suggests that ROS elevation during luteolysis induces luteal cell demise to complete the luteolytic action of PGF.

Summary

The regression of the bovine corpus luteum (CL) is due to the action of endogenous prostaglandin F2α (PGF) released in surge from uterine luminal and glandular epithelial cells at between Day 17 – 19 of the estrous cycle or exogenous PGF given by injection during mid-luteal phase. However, the mechanism of PGF action remains unknown. Based on our current knowledge gained from literature, lifespan and function of CL is protected by endogenous antioxidant enzymes. Thus, it is possible that PGF induced luteolysis by controlling the protective role of antioxidant enzymes. Therefore, in this study we investigated the dynamic change of antioxidant enzymes at the level of protein expression and activity *in vivo* (throughout the estrous cycle and during PGF induced luteolysis) to clarify its possible involvement in the luteolytic action induced by PGF. Bovine corpora lutea were collected at the early (Days 2-3), developing (Days 5-6), mid (Days 8-12), late (Days 15-17) and regressing (Days 19-21) luteal stages (n = 5 CL/stage) and at 0, 2 and 24 h after luteolytic PGF administration (0 h) on Day 10 post ovulation (n = 5 cows/time point). Additional 5 CL were collected at mid-luteal stage and used freshly for immunohistochemical study. CL tissue were dissected from the ovaries and stored at -80°C until analyses of antioxidant enzyme protein expression and activity. Immunohistochemical examination revealed the expression of CAT and GPx1 protein in bovine mid-luteal stage CL tissue, more specifically in large LSCs, small LSCs as well as luteal endothelial cells. The level of SOD1 protein was greater in the developing and mid-luteal stages than in the early, late and regressing-luteal stages. Total SOD activity gradually increased from the early to mid-luteal stages, maintained a high level during the late-luteal stage and then decreased to the lowest level at the regressing-luteal stage. CAT protein expression, CAT and GPx activity increased from the early to mid-luteal stage, then all decreased, reaching their lowest levels at the regressing-luteal stage. The GPx1 protein expression gradually decreased from the developing to the regressing-luteal stage. The GPx1 protein expression level was significantly lower at the regressing-luteal stage than at other stages ($P < 0.05$). During PGF-induced luteolysis, injection of a luteolytic dose of PGF increased luteal SOD1 protein expression, total SOD activity, GPx1 protein expression and GPx activity at 2 h but suppressed them at 24 h. Catalase protein and CAT activity did not change at 2 h but CAT activity decreased ($P < 0.05$) at 24 h. The overall results provide evidence for the protective role of antioxidant enzyme in maintaining CL function during early to late luteal stage in bovine CL. A decrease in these antioxidant

enzymes protein and their activities during regressing luteal stage as well as during structural luteolysis induced by PGF suggests that ROS elevation during luteolysis induces cell demise to complete the luteolytic action of PGF.

CHAPTER 3

MODULATION OF ANTIOXIDANT ENZYMES BY PROSTAGLANDIN F2α AND HYDROGEN PEROXIDE IN CULTURED BOVINE LUTEAL STEROIDOGENIC CELLS *IN VITRO*

Introduction

Corpus luteum (CL) is a small, transient endocrine gland formed following ovulation from the secretory cells of the ovarian follicles [47]. At the mid luteal stage (Days 8 - 12 post ovulation), bovine CL is composed of about 30% luteal steroidogenic cells (LSCs), 53% luteal endothelial cells (LECs), 10% fibrocytes and 7% other cell types [48]. Small LSCs appear to be of thecal cell origin. Large LSCs are of granulosal cell origin [10]. LECs are responsible for vascular formation and play roles in regulating the luteal blood supply [43, 49] whereas LSCs are responsible for P_4 production, the main hormone responsible for the maintenance of pregnancy [50]. CL regression in cattle is initiated by surges of prostaglandin F2α (PGF) secreted from endometrium at between Days 17 – 19 of the estrous cycle (spontaneous luteolysis) [50] or given by injection at mid-luteal phase (exogenous PGF-induced luteolysis) [11]. Despite intensive investigation, the mechanisms by which PGF induces luteal regression remain unclear.

Recent studies showed that treatment of LSCs with PGF induces ROS production and apoptosis [23]. In addition, the CL is exposed to locally produced ROS due to its high blood supply and intensive steroidogenic activity [51]. On the other hand, *in vitro* studies showed that direct treatment of pure populations of luteal steroidogenic cells (LSCs) with PGF does not inhibit basal P_4 production by the large LSCs, and stimulates P_4 production by the small LSCs and by a mixture of large and small LSCs [52, 53] suggesting that PGF action differs in each type of luteal cells or depends on contact between these cells [54].

In chapter 2, our *in vivo* findings showed the evidences for the suppression of ROS defense system (antioxidant enzymes) during regressing stage of the cyclic bovine CL (spontaneous luteolysis) as well as during structural luteolysis induced by exogenous PGF administration, and suggested that ROS elevation during these stages induces cell demise to complete the luteolytic action of PGF. Furthermore, immunohistochemical examination revealed the present of antioxidant enzyme in the both large and small luteal steroidogenic cells. Thus studies on the luteolytic action of PGF-related ROS and antioxidant enzymes in these cells are needed to decode the mechanism action of PGF.

This study aim to clarify possible role PGF and ROS in regulating antioxidant enzymes in bovine CL using cell culture model. Furthermore, the dynamic relationship between PGF and ROS were investigated.

Materials and methods

Determination of prostaglandin F2α (PGF) concentration

The concentration of PGF in the culture medium was determined by enzyme immunoassay (EIA) as described previously [55]. The PGF standard curve ranged from 15.625 to 4000 pg/mL, and the median effective dose (ED50) of the assay was 250 pg/mL. The intra- and inter-assay coefficients of variation were 7.4 and 11.6%, respectively. The cross-reactivities of the antibody were 100% for PGF, 3.0% for PGD2, 1.1% for PGI, 0.15% for PGE2, and < 0.03% for PGA2. The DNA content, estimated using the spectrophotometric method by Labarca & Paigen [56], was used to standardize the PGF concentrations.

Measurement of reactive oxygen species (ROS) production

Bovine luteal cells cultured in 6-well plates containing a collagen coated-coverslip at the bottom were challenged with PGF (1 μM, experimental group) or without PGF (control group) for 2 h and 24 h (n = 5 experiments; each experiment was performed in triplicate). Before the end of the incubation period (30 min, 37°C), a fluorogenic probe for ROS detection (5 μM; CellROX™ Deep Red Reagent; Invitrogen) and cellular nucleus detection (20 μM; NucBlue™ Live Cell Stain; Hoechst 33342, Invitrogen) were added to the culture media in the wells. Then, the culture medium was removed and the cells were washed three times with PBS. The coverslips containing the fluorescent stained cells were used for detection of intracellular ROS. Pictures were taken on an Olympus BX60 fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan; exposure time: 1/80). In each coverslip, 3 microscopic fields were randomly selected. The fluorescent intensities for ROS production across the whole selected microscopic fields were quantified using the image analysis software Adobe Photoshop (Adobe) as described previously [57] with the aid of ImageJ software (Windows version of NIH Image, http://rsb.info.nih.gov/nih-image/, National Institutes of Health). The signal was normalized per unit area.

Results

Effect of hydrogen peroxide (H_2O_2) on prostaglandin F2α (PGF) production in luteal steroidogenic cells cultured for 2 and 24 h

H_2O_2 at concentrations of 10 and 100 µM significantly increased ($P < 0.05$) the concentration of PGF at both 2 h (Fig. 9A) and 24 h (Fig. 9B).

Effect of prostaglandin F2α (PGF) on reactive oxygen species (ROS) production in luteal steroidogenic cells cultured for 2 and 24 h

ROS production in cultured luteal cells was significantly suppressed at 2 h of incubation ($P < 0.05$). However, at 24 h of incubation, ROS production was significantly higher ($P < 0.05$) in the PGF-treated group than in the controls and PGF-treated group at 2 h (Fig. 10B).

Effects of prostaglandin F2α (PGF) and reactive oxygen species on superoxide dismutase (SOD)-1 expression and total SOD activity in cultured luteal steroidogenic cells

PGF and H_2O_2 affected SOD1 protein expression and total SOD activity in a biphasic manner with an increase at 2 h followed by a decrease at 24 h. PGF and H_2O_2 significantly increased SOD1 protein expression (Fig. 11A) and total SOD activity (Fig. 11C) in the short term (2 h), whereas they significantly decreased SOD1 protein expression (Fig. 11B) and total SOD activity (Fig. 11D) in the long term (24 h; $P < 0.05$).

Effects of prostaglandin F2α (PGF) and reactive oxygen species on catalase (CAT) and glutathione peroxidase-1 (GPx1) protein expression, CAT and GPx activity in cultured luteal steroidogenic cells

In LSCs, CAT protein expression (Fig. 12A) and CAT activity (Fig. 13A) did not change while GPx1 protein expression (Fig. 12B) and GPx activity (Fig. 13B) significantly increased at 2 h in cultured LSCs treated with PGF and H_2O_2. Interestingly, PGF and H_2O_2 decreased CAT (Fig. 12C) and GPx1 (Fig. 12D) protein expression, activity of CAT (Fig.13C) and GPx (Fig. 13D) at 24 h in cultured LSCs.

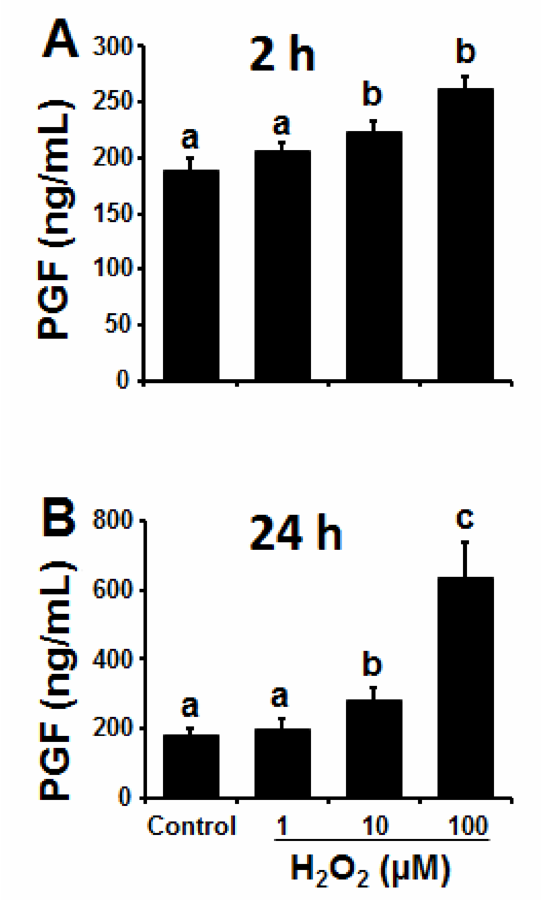

Figure 9. Effect of hydrogen peroxide (H_2O_2) on prostaglandin F2α (PGF) production in cultured bovine cultured luteal steroidogenic cells.

Luteal steroidogenic cells (LSCs) were treated with H_2O_2 (1, 10 or 100 μM) for 2 h (Fig. 9A) or 24 h (Fig. 9B). The concentration of PGF (ng/mL) in the culture medium was assessed by EIA assay. Different superscript letters indicate significant differences ($P < 0.05$) between the control and H_2O_2 treated groups as assessed by ANOVA followed by protected least significant difference test.

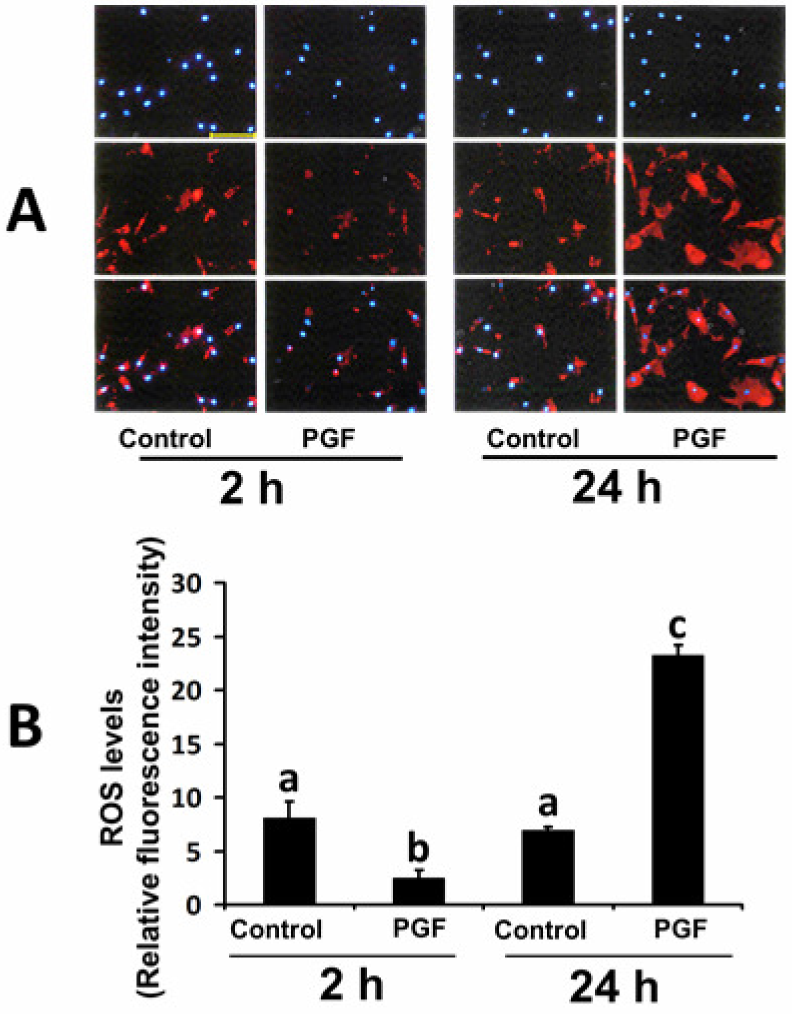

Figure 10. Effect of prostaglandin F2α (PGF) on reactive oxygen species (ROS) production in bovine cultured luteal steroidogenic cells.

Luteal steroidogenic cells (LSCs) were treated with PGF (1 µM) for 2 and 24 h. ROS production was detected by a fluorescence kit (CellROX™ Deep Red Reagent; Invitrogen). Panel "A" shows the representative microscopic field of each group. The scale bar (100 µm) applies to all images. The nuclei appear blue and ROS appear red. The two colors are merged in the bottom of panel "A". Panel "B" shows the result of quantification of ROS. Three macroscopic fields were randomly selected for quantification of ROS production. The red fluorescent signals were quantified using the ImageJ program. Data was expressed as mean ± SEM (n = 5 experiments; each experiment was performed in triplicate). Superscript letters indicate a significant difference ($P < 0.05$) between the control and PGF-treated groups at different time points, as assessed by ANOVA followed by protected least significant difference test.

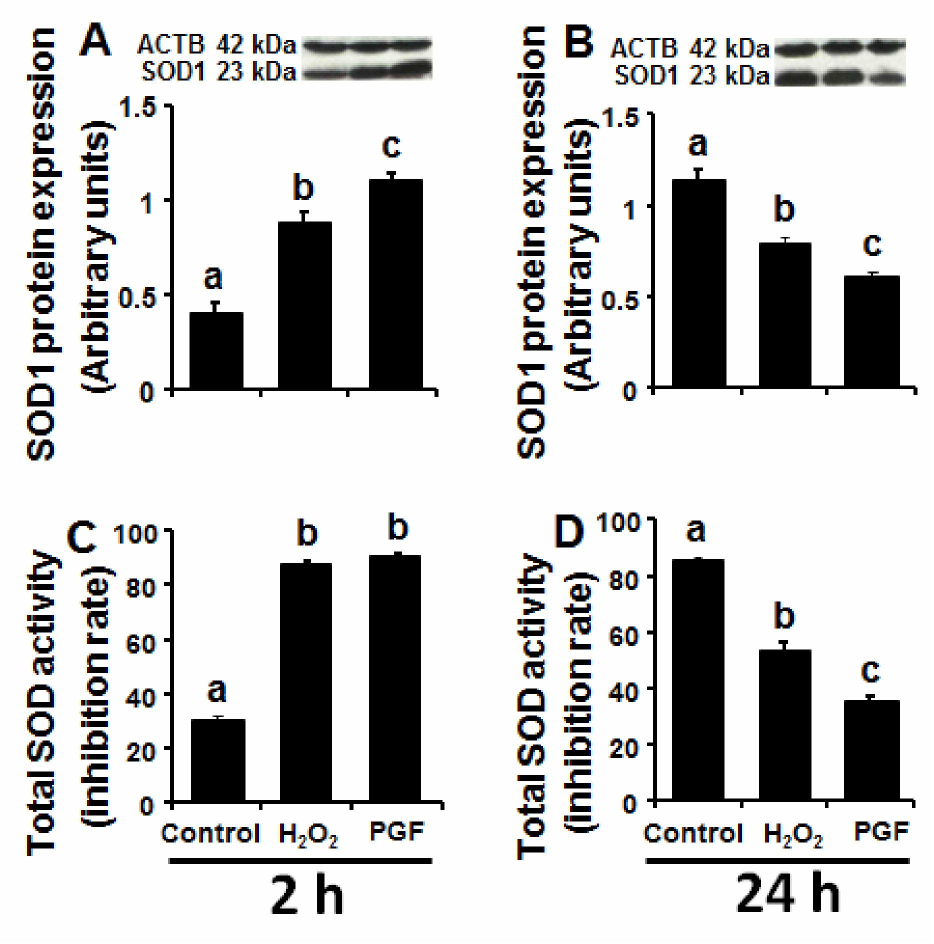

Figure 11. Effect of prostaglandin F2α (PGF) and hydrogen peroxide (H_2O_2) on the expression of superoxide dismutase (SOD)-1 protein expression and total SOD activity in bovine cultured luteal steroidogenic cells.

Biphasic effects of PGF and H_2O_2 on the expression of SOD1 protein (Fig. 11A, B) and total SOD activity (Fig. 11C, D) in bovine luteal cells cultured for 2 (Fig. 11A, C) or 24 h (Fig. 11B, D). Luteal cells were cultured with (experiment groups) or without (control group) PGF (1 μM) or H_2O_2 (10 μM). Different superscript letters indicate significant differences ($P < 0.05$) between the control and experimental groups as assessed by ANOVA followed by protected least significant difference test.

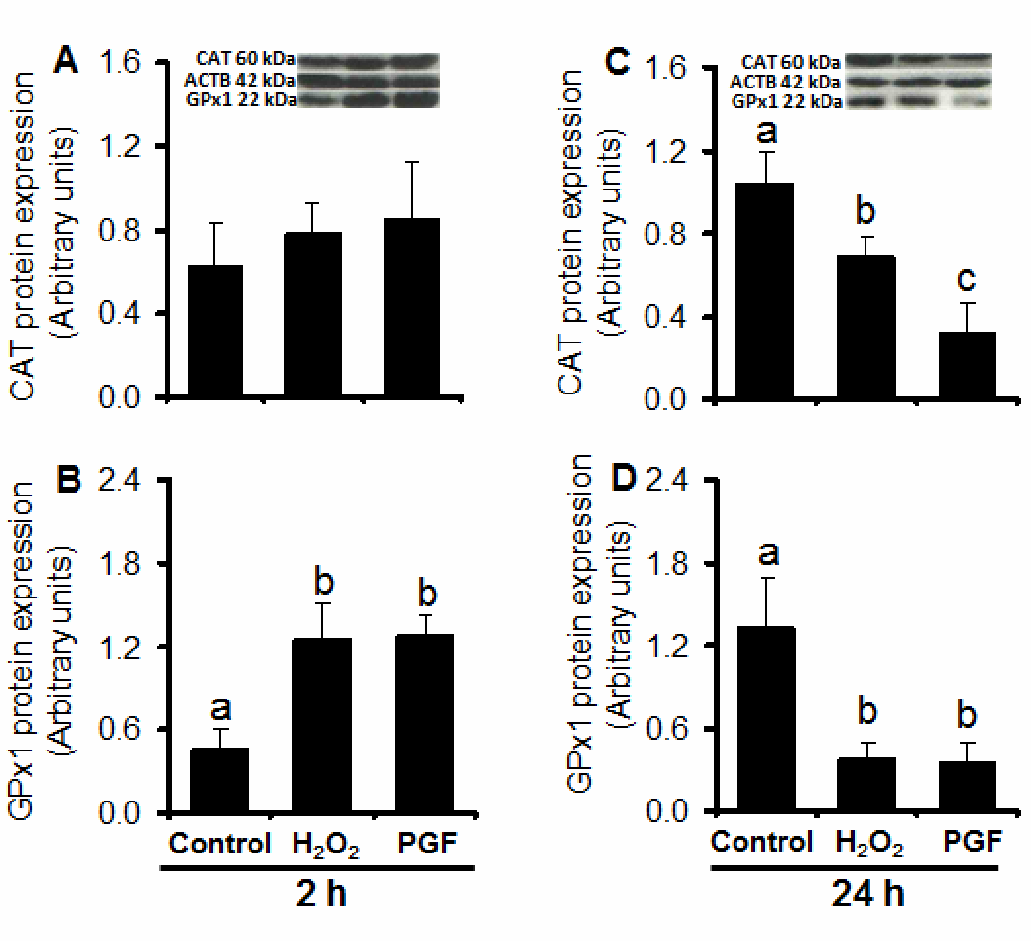

Figure 12. Effects of prostaglandin F2α (PGF) and hydrogen peroxide (H_2O_2) on catalase (CAT) and glutathione peroxide-1 (GPx1) protein expression in bovine cultured luteal steroidogenic cells.

Bovine cultured luteal cells were exposed to PGF (1 μM) or H_2O_2 (10 μM) for 2 (mimicking functional luteolysis) and 24 h (mimicking structural luteolysis). Catalase protein expression (Fig. 12A, C), GPx1 protein expression (Fig. 12B, D) in cultured cells were examined by western blotting. Data are the mean ± SEM (n = 5 experiments, in each treatment, the cells were cultured in triplicate). Representative samples of Western blot for CAT, GPx1 and ACTB (internal control) are shown in the upper panel of Fig. 12A and 12C. Different superscript letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by protected least significant difference test.

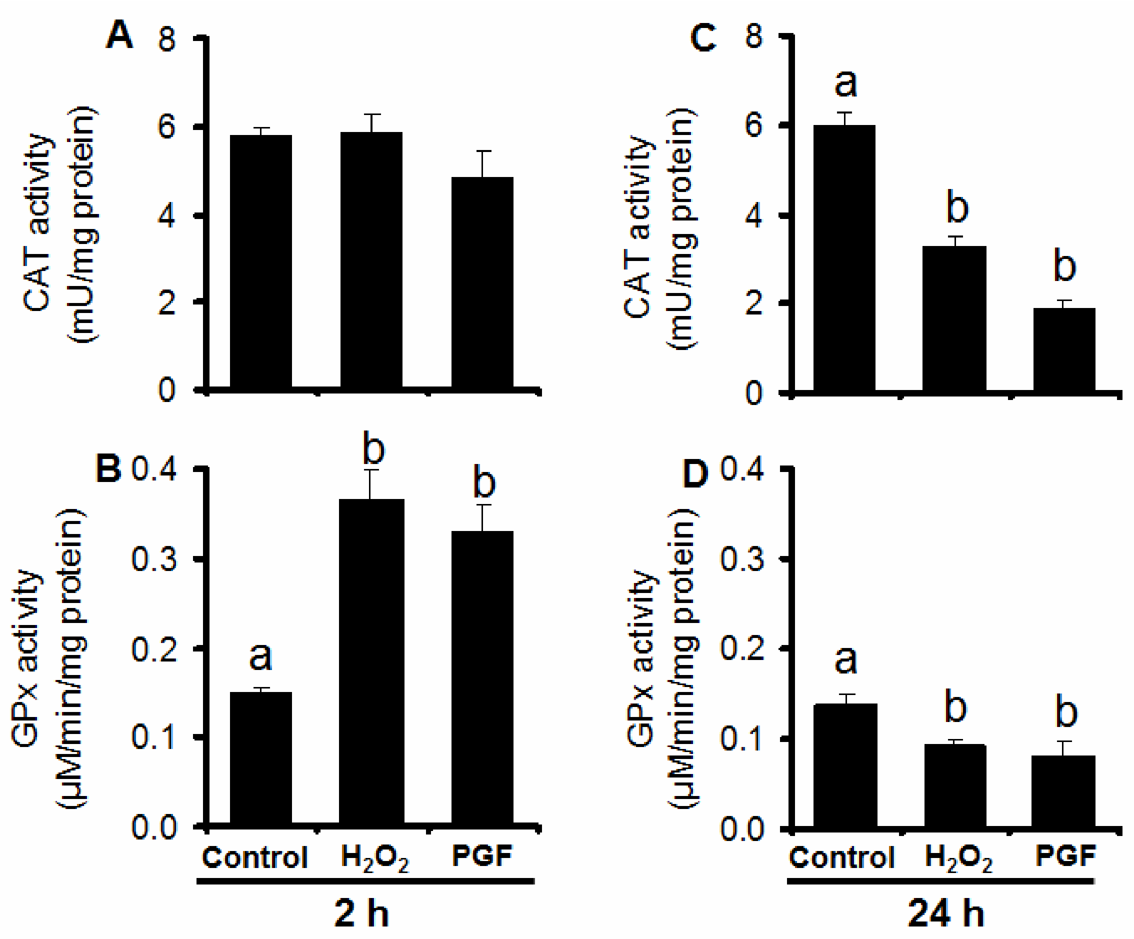

Figure 13. Effects of prostaglandin F2α (PGF) and hydrogen peroxide (H_2O_2) on catalase (CAT) and glutathione peroxidase (GPx) activity in bovine cultured luteal steroidogenic cells.

Bovine cultured luteal cells were exposed to PGF (1 µM) or H_2O_2 (10 µM) for 2 (mimicking functional luteolysis) and 24 h (mimicking structural luteolysis). CAT activity (Fig. 13A, C) and GPx activity (Fig. 13 B, D) in cultured cells were determined by colorimetric method using commercial assay kit (CAT assay kit, Bio Vision; GPx assay kit, Cayman). Different superscript letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by protected least significant difference test.

Discussion

Luteal steroidogenic cells (LSCs) are responsible for P_4 production, the main hormone responsible for the maintenance of pregnancy [50]. A rapid decrease in plasma P_4 concentration was observed during PGF-induced luteolysis in cows [13]. In addition, LSCs produce PGF [58-60] and ROS [19, 61] and express PGF receptors [10, 39]. In the present study, PGF and H_2O_2 decreased SOD1, CAT and GPx1 protein expression and activity at 24 h in cultured luteal cells. These findings seem to be consistent with our *in vivo* study in which SOD, CAT and GPx decreased 24 h post-luteolytic PGF treatment. These findings suggest that LSCs are targets of the luteolytic action of PGF and that PGF induces luteolysis by regulating antioxidant enzymes in LSCs.

Surprisingly, CAT protein expression and CAT activity did not change while SOD1 protein expression, GPx1 protein expression, total SOD activity and GPx activity significantly increased at 2 h in cultured LSCs treated with PGF and H_2O_2. These results indicate that PGF may differently regulate SOD, CAT and GPx. The reason for the transient increases in SOD and GPx after exposure of the cultured cells to PGF and H_2O_2 is unknown. It is possible that acute elevation of antioxidant enzymes represent a response of luteal cells to protect themselves against the cellular damage induced by PGF during functional luteolysis.

Although luteolytic PGF is derived from the uterus in many species, including ewes [62] and cows [9], a considerable amount of PGF is also synthesized by the CL [36]. ROS has been demonstrated to stimulate PGF production in the CL of rats [63], cows [36] and human [64]. In turn, PGF induces ROS generation the ovine [22] and rat [23] CL. Interestingly, in the present study, H_2O_2 stimulated PGF production in cultured bovine LSCs at both 2 and 24 h and PGF induced generation of ROS at 24 h *in vitro*. The above findings suggest the presence of a positive feedback loop between PGF and ROS in the bovine CL, more specifically in LSCs during luteolysis. Also, the increase of intraluteal PGF induced by ROS seems to be crucial for promotion of luteal regression in cow.

PGF reduced luteal blood flow by stimulating vasoactive substances such as endothelin (ET-1) and angiotensin (Ang II; [65]. Decreasing the blood supply to the CL not only reduces the nutrient supply but also creates a low oxygen condition (hypoxia) for the luteal cells. Hypoxia induces ROS generation [66, 67] by activating the xanthin-xanthin oxidase system [19]. The produced ROS in turn induce PGF production by stimulating phospholipase 2 and COX, the enzymes responsible for PGF biosynthesis from arachidonic acid [68]. In the present study, H_2O_2 increased the production of PGF by bovine cultured LSCs at both 2 and 24 h after treatment. This result suggests that the increase in ROS production during structural luteal regression might be part of the mechanism responsible for inducing luteal production of PGF. Furthermore, PGF significantly increased the production of ROS at 24 h but decreased it at 2 h of incubation. The suppression of ROS production is likely due to the increase in antioxidant enzymes expression and activity in cultured luteal cells at 2 h after PGF treatment, whereas the increase in ROS production is likely due to decreased SOD, CAT and GPx expression and activity at 24 h after PGF treatment. The decrease in antioxidant enzymes may be due to the accumulative luteolytic effect of PGF produced by the stimulation of ROS, which consequently results in an excessive increase in intraluteal ROS concentration, causing luteal cell demise.

In addition, SOD convert O_2^- into H_2O_2, a type of ROS which also causes cell death [69] through up-regulation of the death receptor (Fas). Then, H_2O_2 is converted to water and oxygen by catalase (CAT) or glutathione peroxidase (GPx) [32]. Therefore, the single increase in SOD without elevation of CAT or GPx may enhance the accumulation of H_2O_2. In cultured luteal cells at 2 h, ROS production decreased while SOD1 expression and activity increased together with the increase of GPx. This suggests that GPx may take more important role than CAT in suppressing the increase of H_2O_2 generated by the elevation of SOD. Our findings about the change of luteal antioxidant enzymes in LECs [3] and LSCs suggest that the biphasic regulation of antioxidant enzymes by PGF is a complex process happening in different components of the CL. These findings provide complementary information to understand how luteal antioxidant enzymes are regulated during endogenous and exogenous PGF-induced luteolysis in cows.

In conclusion, the present study provides evidence that the interaction between PGF and ROS could either increase or decrease antioxidant enzymes expression and activity in cultured luteal cells according to the time of exposure. These findings confirmed that LSCs are targets of the luteolytic action of PGF, and that PGF in interaction with ROS induced luteolysis by suppressing antioxidant enzymes in LSCs only during structural luteolysis but not during functional luteolysis.

Summary

Antioxidant enzymes play important roles in maintaining the corpus luteum function by reducing the cellular damage induced by reactive oxygen species (ROS). Prostaglandin F2α (PGF) is well known as a physiological luteolysin. However, cellular events associated with luteolysis remain poorly characterized. In the present *in vitro* study, the dynamic relationship between PGF and ROS as well as its possible role in regulating antioxidant enzymes in bovine CL using cultured bovine luteal cells were examined to clarify the mechanism of action of PGF during luteolytic process. Luteal steroidogenic cells (LSCs) isolated from CL tissue at mid-luteal stage (Days 8-12 of the estrous cycle) were treated with PGF and H_2O_2 for 2 h (mimicking functional luteolysis) or 24 h (mimicking structural luteolysis). H_2O_2 stimulated PGF biosynthesis at 2 and 24 h in a dose- and time-dependent manner. PGF, in turn, induced ROS production. PGF (1 μM) and H_2O_2 (10 μM) increased SOD1 protein expression and total SOD activity, GPx1 protein and GPx activity at 2 h ($P < 0.05$) but suppressed them at 24 h ($P < 0.05$). CAT protein expression and activity did not change at 2 h but they were suppressed at 24 h by PGF and H_2O_2 ($P < 0.05$). These findings confirmed that LSCs are targets of the luteolytic action of PGF and that PGF in interaction with ROS induced luteolysis by suppressing antioxidant enzymes in LSCs only during structural luteolysis but not during functional luteolysis.

GENERAL CONCLUSION

The present study aims to clarify the roles of antioxidant enzymes in regulating the luteolytic action of prostaglandin F2α (PGF) and reactive oxygen species (ROS). The overall results demonstrated that PGF through its interaction with ROS regulates the expressions and the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), in bovine corpus luteum (CL), more specifically in luteal steroidogenic cells (LSCs), suggesting that these enzymes are involved in the mechanism of action of PGF in bovine CL. The down-regulation of these proteins and their activities during structural luteolysis could enhance the accumulation of reactive oxygen species, which would result in both increasing luteal PGF production and oxidative stress, to complete the CL regression in cattle. Based on the findings from present and previous studies [22, 23, 36, 68, 70] we propose a model integrating PGF, luteal antioxidant enzymes and ROS production during the time of functional (2 h) and structural (24 h) luteolysis (Fig. 14).

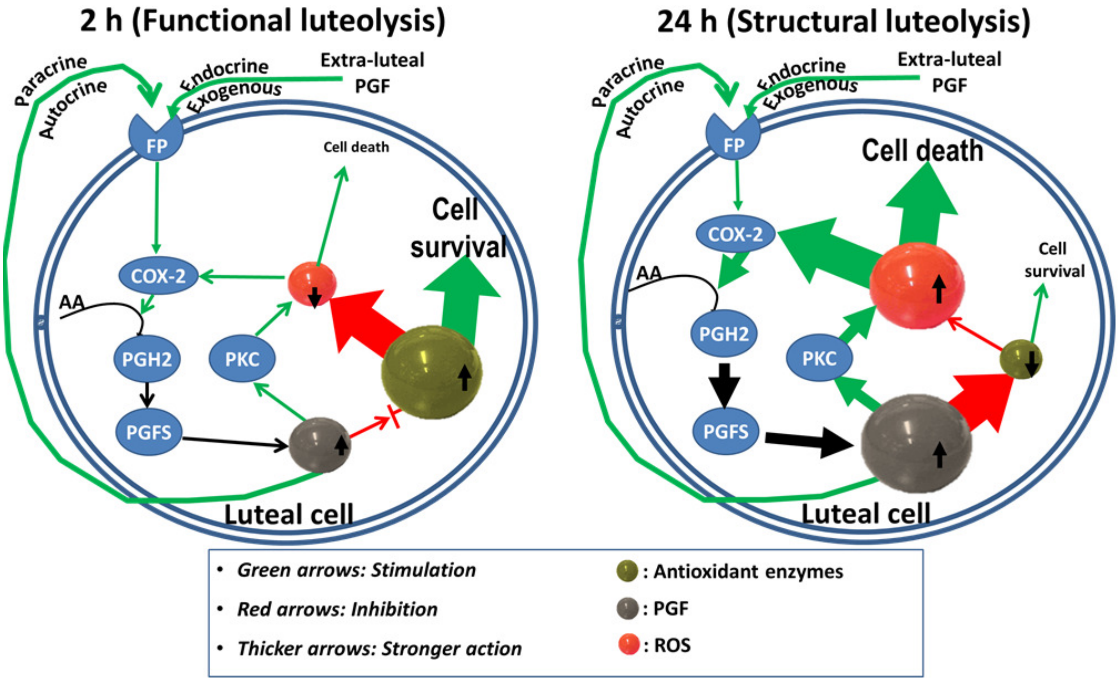

Figure 14. Working model of the interaction between exogenous prostaglandin F2α (PGF), uterine PGF, luteal PGF, luteal antioxidant enzymes and reactive oxygen species (ROS) production.

At 2 h: Extra luteal PGF binds to PGF receptor (FP) present in luteal cells and activates COX-2, an enzyme responsible for PGF synthesis by inducing the conversion of arachidonic acid (AA) into prostaglandin H2 (PGH2). Produced luteal PGF from PGH2 induces ROS production through activating protein kinase C (PKC) and up-regulate luteal SOD, GPx protein expression and activity. The generated ROS in turn induces COX-2. ROS cause cell death by apoptosis. Since antioxidant enzymes are up-regulated at 2 h, antioxidant enzymes could be able to reduce the accumulation of ROS and therefore rescue the luteal cell from demise. At 24 h: The positive feedback loop between PGF and ROS remains while antioxidant enzymes are down-regulated by PGF. That consequently enhances ROS accumulation. When the accumulation of ROS is over the luteal protective capacity of antioxidant enzymes, death of luteal cells and structural luteolysis occurs. Locally generated PGF may also act in a paracrine/autocrine manner.

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