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Catalase and glutathione peroxidase expression in bovine corpus luteum during the estrous cycle and their modulation by prostaglandin F2*α* and H_2O_2

H.V. Vu, T.J. Acosta[1]

Laboratory of Reproductive Physiology, Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan.

Abstract

Antioxidant enzymes seem to play roles in controlling the luteal function and the luteolytic action of prostaglandin F2α (PGF). The aim of this study was to clarify the roles of catalase (CAT) and glutathione peroxidase (GPx) in the luteolytic action of PGF in both corpus luteum (CL) and cultured luteal cells. Corpora lutea were collected at the early (days 2-3), developing (days 5-6), mid (days 8-12), late (days 15-17) and regressed (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 (n = 5 cows/time point). Catalase protein and the activities of CAT and GPx increased from the early to mid-luteal stage, then all decreased ($P < 0.05$), reaching their lowest levels at the regressed luteal stage. The levels of GPx1 protein were lower in the regressed luteal stage than in other stages ($P < 0.05$). Immunohistochemical examination also revealed the expression of CAT and GPx1 protein in the bovine CL tissue. Injection of a luteolytic dose of PGF increased luteal GPx1 protein and GPx activities 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. Prostaglandin F2α (1 μM) and H_2O_2 (10 μM) decreased CAT and GPx1 protein expression and activity at 24 h in cultured luteal cells isolated from mid-luteal stage CL (n = 3 CL per each experiment). Interestingly, CAT protein and activity did not change while GPx1 protein and activity increased at 2 h in luteal cells treated with PGF and H_2O_2 ($P < 0.05$). The down-regulation of CAT and GPx, and their activities during structural luteolysis might enhance the accumulation of reactive oxygen species, which would result in both increasing luteal PGF production and cell death to complete CL regression in cattle.

Keywords: bovine corpus luteum, catalase, glutathione peroxidase, luteolysis, prostaglandin F2α.

Introduction

The corpus luteum (CL) forms in the ovary after ovulation and produces progesterone (P_4), the hormone responsible for the maintenance of pregnancy (Skarzynski *et al*., 2009). If pregnancy does not occur, the CL regresses and loses its capacity to produce P_4 (Agarwal *et al*., 2012). 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 (Gibson, 2008).

Prostaglandin F2α (PGF) is a luteolytic factor in mammals. In the cow, both endogenous PGF synthesized by the uterus at the late-luteal stage (McCracken *et al*., 1999) and exogenous PGF given during the mid-luteal stage (Schallenberger *et al*., 1984) 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; Juengel *et al*., 1993; Acosta *et al*., 2002). In addition, in the cow (Pate, 1988) and ewe (Rexroad and Guthrie, 1979; Lee *et al*., 2012) the CL also synthesizes PGF. Nevertheless, 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 (Garrel *et al*., 2007). Reactive oxygen species include superoxide radicals, hydrogen peroxide and hydroxyl radicals (Kato *et al*., 1997). Antioxidant enzymes control the ROS concentration. The balance between ROS generation and ROS elimination by antioxidant enzymes helps maintain cellular function. An increase in intracellular ROS levels by an increase in ROS production or a decrease in antioxidant enzyme levels can lead to cell death (Garrel *et al*., 2007) by apoptosis (Juengel *et al*., 1993; Buttke and Sandstrom, 1994). Reactive oxygen species have been implicated in the regulation of luteal function, including luteo-protective (Kawaguchi *et al*., 2013) and luteolytic roles (Riley and Behrman, 1991b; Carlson *et al*., 1993; Tanaka *et al*., 2000; Hayashi *et al*., 2003; Al-Gubory *et al*., 2010; Taniguchi *et al*., 2010). Reactive oxygen species generation is induced by PGF in the ovine (Hayashi *et al*., 2003), rat (Tanaka *et al*., 2000) and bovine CL (Vu *et al*., 2013). Intraluteal ROS increases during luteal regression (Riley and Behrman, 1991b; Shimamura *et al*., 1995) and it has the capacity to inhibit P_4 production (Sawada and Carlson, 1991; Sugino *et al*., 1993). In addition, we and others found that PGF production is induced by ROS in bovine luteal

[1]Corresponding author: acosta@cc.okayama-u.ac.jp
Phone: +81(86)251-8349; Fax: +81(86)251-8349
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cells and that ovarian ROS production is rapidly inducible by PGF *in vitro* (Margolin and Behrman, 1992; Vu *et al*., 2013) and *in vivo* (Acosta *et al*., 2009). However, the effects of ROS on the CL may differ depending on time or dose (Vega *et al*., 1995). Treatment of human mid luteal cells with a low concentration of hydrogen peroxide (H_2O_2; 0.01 μM) significantly stimulated P4 secretion while treatment with a higher concentration (100 μM) markedly inhibited human chorionic gonadotropin (hCG)-stimulated P_4 and estradiol secretion (Vega *et al*., 1995).

Since the local ROS concentrations are controlled by antioxidant enzymes, it is possible that these enzymes are involved in regulating the luteolytic action of PGF (Minegishi *et al*., 2002). Antioxidant enzymes include superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidases (GPx). Superoxide dismutases are located in the nucleus and cytoplasm (type 1), in the mitochondria (type 2) and in the extracellular matrix (type 3). Catalase is usually located in a cellular organelle called the peroxisome (Mueller *et al*., 2002). Glutathione peroxidases include several isozymes that differ according to their cellular location and substrate specificity (Muller *et al*., 2007). Glutathione peroxidase type 1 (GPx1), the most abundant type, scavenge H_2O_2 in the cytoplasm (Muller *et al*., 2007). All of these antioxidant enzymes are found in nearly all living organisms exposed to oxygen (Chelikani *et al*., 2004). Superoxide dismutases convert oxygen radicals into H_2O_2. H_2O_2 is then converted to water and oxygen by CAT (Chelikani *et al*., 2004) and GPx (Al-Gubory *et al*., 2008). Therefore, an increase of SOD without elevation of CAT or GPx may enhance the accumulation of H_2O_2. The down-regulation of glutathione peroxidase causes bovine luteal cell apoptosis during structural luteolysis (Nakamura *et al*., 2001). In bovine CL, the expression of CAT and SOD are low during luteal regression, which suggests that oxidative stress has a role in inducing luteolysis (Rueda *et al*., 1995). Our previous study (Vu *et al*., 2012) found that the SOD expression and activity increased during functional luteolysis. The increase of SOD could induce different effects in luteal cells depending on the levels of CAT and GPx: 1) SOD protects the luteal cells when it increases concomitantly with CAT and/or GPx; 2) SOD enhances cell death when it increases without elevation of CAT or GPx to scavenge H_2O_2 (Vu *et al*., 2012). Thus, CAT or GPx seem to control luteal function by regulating the intraluteal actions of PGF. The aim of this study was to clarify the role of CAT and GPx on the luteolytic action of PGF in both CL and cultured luteal cells.

Materials and Methods

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 (Okuda *et al*., 1988; Miyamoto *et al*., 2000; Vu *et al*., 2012). 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 protein and enzyme activity analyses.

Collection of bovine corpus luteum tissues during PGF-induced luteolysis

The collection procedures were approved by the local institutional animal care and use committee of 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 (Dinoprost, Dinolytic; Pharmacia & Upjohn, Belgium) with an 11-day interval according to the manufacturer's direction. Ovulation was determined by a veterinarian via transrectal ultrasonographic examination. Then, corpora lutea were collected with 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 (Dinoprost, Dinolytic; Pharmacia; 0 h), and at 2 and 24 h post-treatment (n = 5 cows per time point). Corpus luteum tissues were dissected from the ovaries and then immediately stored at -80°C until protein and enzyme activity analyses.

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 (day 8-12). Luteal cells were obtained as described previously (Okuda *et al.*, 1992). 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 6-ml culture flasks (no. 658175; Greiner Bio-One, Frickenhausen, Germany) for determining CAT and GPx protein expression or CAT and GPx activity. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 38°C in an N_2- O_2- CO_2-regulated incubator (no. BNP-110; ESPEC CORP., Osaka, Japan). 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 (1 µM) or H_2O_2 (10 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 (Vu *et al*., 2012). After 2 h (mimicking functional luteolysis) or 24 h (mimicking structural luteolysis) of incubation, the cultured cells and/or medium were collected and used immediately or stored at -80°C until further analysis.

CAT and GPx protein expression

Protein expressions for CAT and 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*. (1993), 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 Immunochemicals 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 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 bands 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.

CAT and GPx activity assay

CAT activity in CL tissue or in cultured cells at the end of the 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 x 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 CAT activity was normalized to milligram of protein used in the assay and was expressed as mU/mg protein.

GPx activity in CL tissue or in cultured cells at the end of the 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 is described in the user's manual included in the kit. Results are presented as micro mol/min/mg protein. Mainly, 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 (Ismail *et al*., 2010). Glutathione peroxidase activity was expressed as micromoles of NADPH oxidized. The results were normalized to milligram of protein used in the assay.

Localization of CAT and GPx1 protein by immunohistochemistry.

Bovine corpus luteum tissues at mid-luteal stage (day 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 a 1 h incubation with primary CAT antibody (Anti-Catalase [Bovine liver] Rabbit [1:300 dilution; no. 200-4151; Rockland Immunochemicals Inc., Gilbertsville, PA, USA]) or GPx1 (Rabbit polyclonal antibody, anti-Glutathione peroxidase 1 [1:300 dilution; no. ab22604; Abcam]). Following incubation at room temperature, sections were washed in PBS, incubated with immPRESS™ 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 cell cytoplasm, 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.

Statistical analysis

Data from the CAT and GPx1 protein level, and 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 CAT and GPx1 protein, or CAT and GPx activity, 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 significantly different when P value is less than 0.05.

Results

Localization of CAT and GPx1 protein by immunohistochemistry

Immunohistochemical examination revealed

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

In experiment 1, during the estrous cycle, CAT protein expression (Fig. 3A) and the activity (Fig. 3C) and GPx activity (Fig. 3D) increased from the early to mid luteal stage, then all decreased ($P < 0.05$), reaching their lowest levels at the regressed luteal stage. The GPx1 protein expression gradually decreased from the developing to the regressed luteal stage (Fig. 3B). The GPx1 protein expression level was significantly lower at the regressed luteal stage than at other stages ($P < 0.05$) ($n = 5$ CL per stage). An injection of a luteolytic dose of PGF significantly increased luteal GPx1 protein expression (Fig. 4B) and GPx activities (Fig. 4D) at 2 h but suppressed it at 24 h. Catalase protein expression (Fig. 4A) and CAT activity (Fig. 4C) did not change at 2 h but CAT activity significantly decreased ($P < 0.05$) at 24 h. In experiment 2, CAT protein expression (Fig. 5A) and CAT activity (Fig. 5C) did not change while GPx1 protein expression (Fig. 5B) and GPx activity (Fig. 5D) significantly increased at 2 h in cultured LSCs treated with PGF and H_2O_2. Interestingly, PGF and H_2O_2 decreased CAT (Fig. 5E) and GPx1 (Fig. 5F) protein expression, activity of CAT (Fig. 5G) and GPx (Fig. 5H) at 24 h in cultured LSCs.

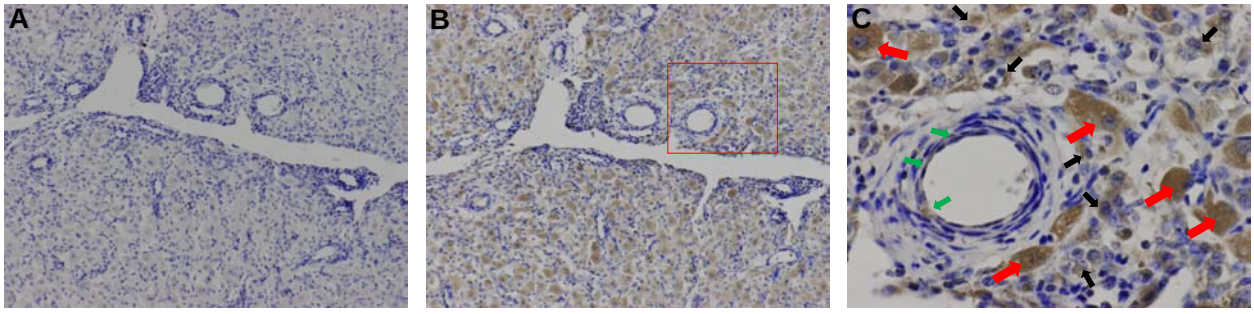

Figure 1. Representative images of immunohistochemical expression of CAT in corpora lutea from cycling cow. Images A and B show sections of luteal tissue with negative and positive CAT expression, respectively (magnification: 200X). Image C is part of image B at higher magnification (400X). The arrows show examples of large LSCs (red arrows), small LSCs (black arrows) as well as LECs (green arrows) expressing the CAT protein.

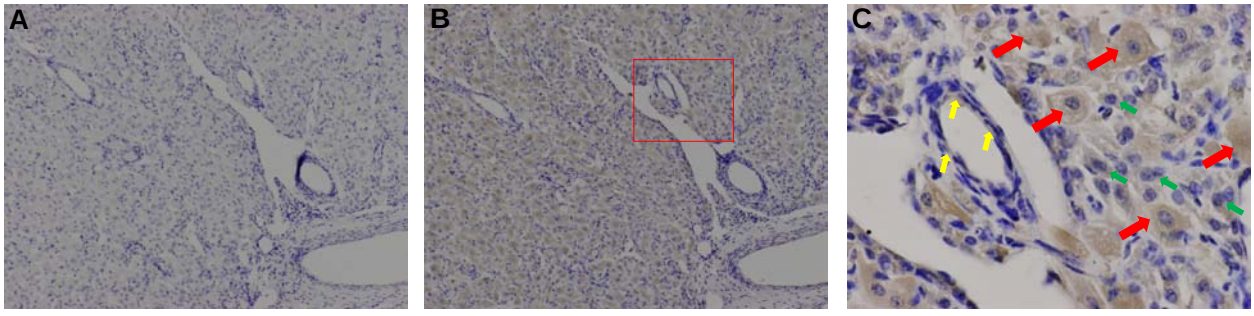

Figure 2. Immunohistochemistry of GPx1 in bovine luteal tissue. Immunohistochemical representative pictures of GPx1 were shown. Picture A is negative control while picture B is positive staining. Image C is part of image B at higher magnification (400X). The arrows show examples of large LSCs (red arrows), small LSCs (green arrows) and LECs (yellow arrows) expressing the GPx1 protein.

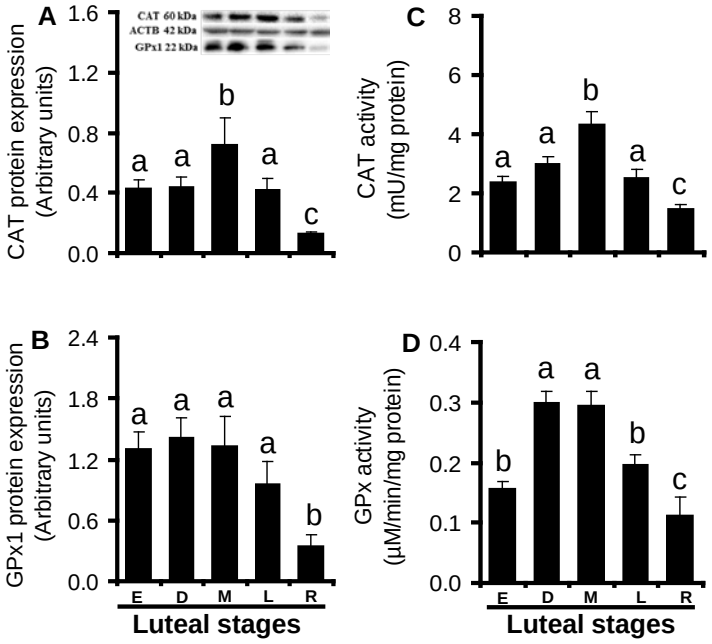

Figure 3. Changes of catalase (CAT) and glutathione peroxide (GPx) in luteal tissue throughout the luteal stages (early [E], days 2-3; developing [D], days 5-6; mid [M], days 8-12; late [L], days 15-17; regressed [R], days 19-21). Data are the mean ± SEM for five samples per luteal stage. Catalase protein 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 panel A. The enzyme activity of CAT (C) and GPx (D) 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.

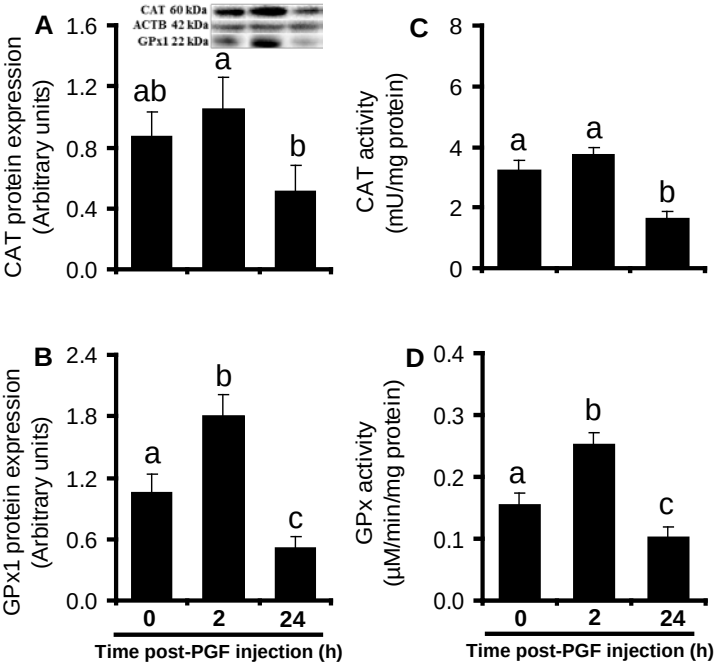

Figure 4. Changes in CAT and GPx in luteal tissue during PGF induced-luteolysis. Bovine CL tissue collected just before (0 h, control) and after administration (2 h, 24 h) of luteolytic dose of PGF. Protein expressions of CAT (A) and GPx1 (B) were assessed by Western blot. Representative samples of Western blot for CAT, GPx1 and ACTB (internal control) are shown in the upper panel of Fig. 4A. 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.

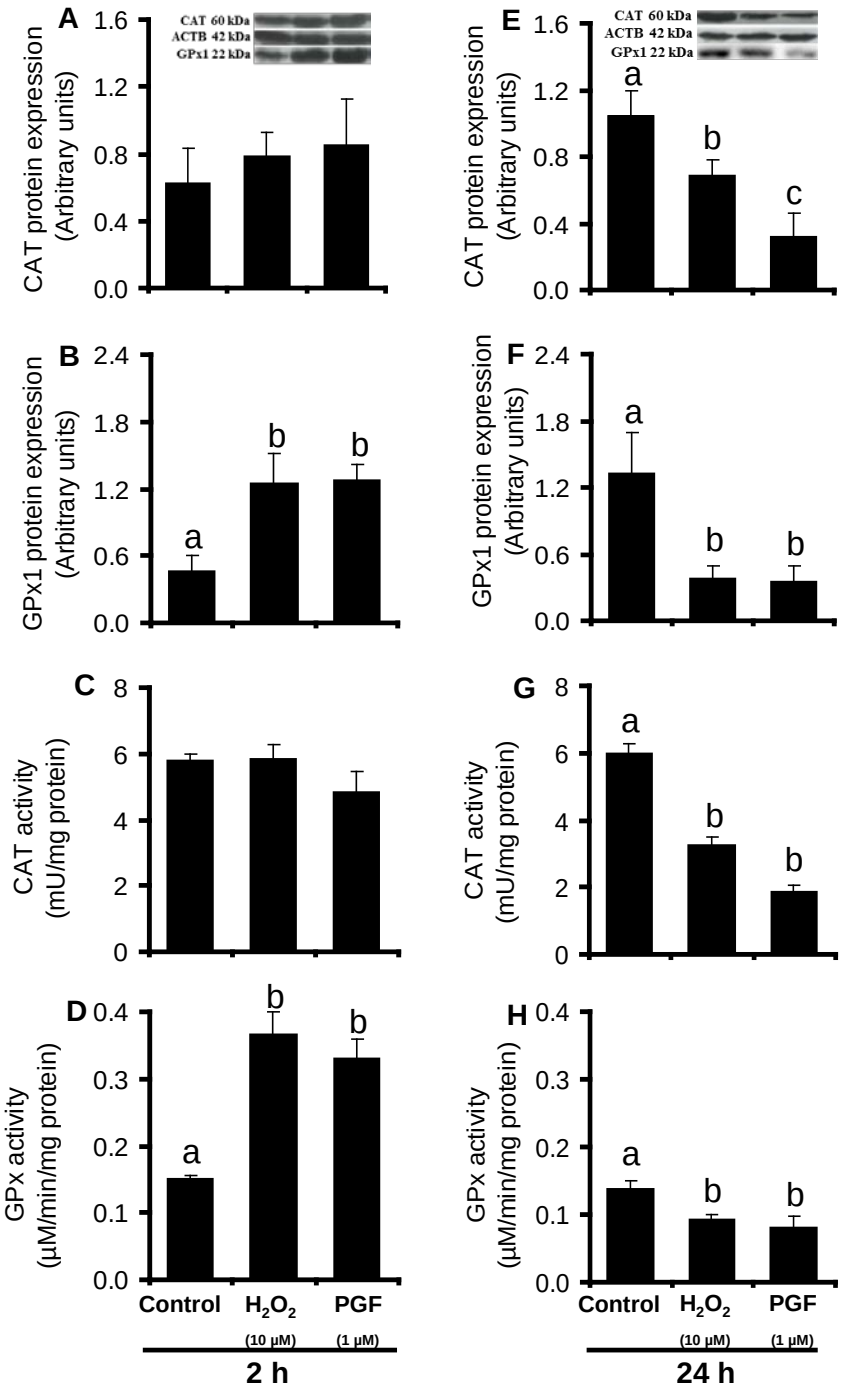

Figure 5. Effects of PGF and H_2O_2 on CAT and GPx1 protein expression, CAT and GPx activity in LSCs cultured for 2 h (mimicking functional luteolysis) and 24 h (mimicking structural luteolysis). Bovine cultured luteal cells were exposed to PGF (1 µM) or H_2O_2 (10 µM) for 2 and 24 h. Catalase protein expression (A, E), GPx1 protein expression (B, F), CAT activity (C, G) and GPx activity (D, H) in cultured cells were examined by western blotting and activity assay kits, respectively. 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. 5A and 5E. Catalase and GPx activity were determined by colorimetric method using a 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

The present study demonstrated that the catalase (CAT) and glutathione peroxidase (GPx) are expressed in bovine luteal tissues. The expression and activity of 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, together with our previous findings on the increase in production of reactive oxygen species (ROS) and the decrease in superoxide dismutase (SOD) during structural luteolysis (Vu *et al*., 2013), 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, spontaneous luteal regression is induced by the episodic PGF pulse secreted from the endometrium between days 17 and 19 of the estrous cycle (McCracken *et al*., 1999). Luteal superoxide dismutase (SOD) was regulated by PGF *in vivo* and *in vitro*, suggesting that antioxidant enzymes mediate the luteolytic action of PGF (Vu *et al*., 2012, 2013). An increase in SOD results in up-generation of H_2O_2 because H_2O_2 is the product of conversion of oxygen radical (O_2^-) by SOD. H_2O_2, a secondary type of ROS, is still toxic. It is also more stable than O_2^- and can pass through cell membranes more easily than O_2^-. H_2O_2 can also react with O_2^- to form the OH^- ion, which is more toxic than H_2O_2 and O_2^- (Liu *et al*., 2001). Thus, CAT and GPx are needed to convert SOD-produced H_2O_2 to a non toxic substance (water), to protect the cell from the negative effects of ROS. In the present study, we found that the protein expression and activity CAT and GPx concomitantly increased from the early to mid luteal phase, suggesting that the balance between antioxidant enzymes and ROS leans to antioxidant enzymes. In other words, antioxidant enzymes can help the cells overcome the detrimental effect of ROS and the CL keeps its function 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*. (1995) 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 by Nakamura *et al*. (2001), in which CAT was highly expressed in the middle stages of the estrous cycle.

By contrast, during the regressing luteal stage in which PGF has a luteolytic effect (Arosh *et al*., 2004), both CAT and GPx protein expression and activity decreased to the lowest level. Rapoport *et al*. (1998) found that CAT activity decreased concomitantly with the decrease in P4 during the regressing stage of bovine estrous cycle. In addition, Nakamura *et al*. (2001) 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. Overall, these findings strongly support the hypothesis that PGF induces luteal regression by suppressing the protective role of antioxidant enzymes in the bovine corpus luteum.

Exogenous PGF given in the mid luteal stage induces functional luteolysis (until 12 h post PGF injection) and structural luteolysis (24 h post PGF injection; Neuvians *et al*., 2004; Schams and Berisha, 2004; Berisha *et al*., 2010). The mechanisms involved in the luteolytic action of PGF are complex and poorly understood. Luteolysis involves the participation of reactive oxygen species (ROS; Schams and Berisha, 2004; Agarwal *et al*., 2005, 2012). Reactive Oxygen Species have been reported as a trigger of functional luteolysis (Sawada and Carlson, 1989; Riley and Behrman, 1991a, b). The level of ROS increases in the regressing CL of rats (Sugino *et al*., 1993; Shimamura *et al*., 1995). Reactive Oxygen Species cause cell death by apoptosis (Noda *et al*., 2012), which occurs during both spontaneous and PGF-induced luteal regression in cattle (Juengel *et al*., 1993; McCracken *et al*., 1999). The synthesis of steroid hormone is accompanied by formation of ROS (Rapoport *et al*., 1998) and PGF increases the production of ROS in cows (Acosta *et al*., 2009) and rats (Riley and Behrman, 1991a; Tanaka *et al*., 2000). In turn, Reactive Oxygen species stimulate PGF production (Nakamura and Sakamoto, 2001; Sander *et al*., 2008; Vu *et al*., 2013). These observations support the concept that ROS mediate the luteolytic cascade induced by PGF and that antioxidant enzymes are involved in the mechanism of PGF action during luteolysis by regulating antioxidant enzymes. In the present study, both CAT and GPx1 protein expression and activity of GPx and CAT were down regulated by PGF treatment at the regressed luteal stage. These findings support the concept that suppression of antioxidant enzymes during PGF induced-luteolysis results in excessive increase in ROS formation, leading to the demise of luteal cells.

CL is one of the major endocrine substructures of the ovary (Harold *et al*., 1997). The CL is exposed to locally produced ROS due to its high blood supply and intensive steroidogenic activity (Al-Gubory *et al*., 2012). The CL is composed of different types of cells. At the mid luteal stage (day 12 post ovulation), bovine CL is composed of about 30% LSCs, 53% luteal endothelial cells (LECs), 10% fibrocytes and 7% other cell types (O'Shea *et al*., 1989). Luteal endothelial cells are responsible for vascular formation and play roles in regulating the luteal blood supply (Davis *et al*., 2003; Vu *et al*., 2013) whereas LSCs are responsible for P4 production, the main hormone responsible for the maintenance of pregnancy (Diaz *et al*., 2002). A rapid decrease in plasma P4 concentration was observed during PGF-induced luteolysis in cows (Acosta *et al*., 2002). Although PGF is well known as a physiological luteolysin, cellular events associated with luteolysis remain poorly characterized. In the present *in vitro*

study, we examined the response of LSCs exposed to PGF and H_2O_2. An unexpected finding was that GPx protein expression and activity were up regulated 2 h post luteolytic PGF injection. This acute increase in antioxidant enzymes after injection of a luteolytic dose of PGF may be an adaptive response of the CL to oxidative stress induced by PGF (Garrel *et al*., 2007), or might be due to the activation of the neuro-endocrine stress axis in vivo (Vu *et al*., 2013).

LSCs produce PGF (Milvae and Hansel, 1983; Rodgers *et al*., 1988; Hu *et al*., 1990) and ROS (Kato *et al*., 1997; Hanukoglu, 2006) and express PGF receptors (Arosh *et al*., 2004). Our previous study found that SOD1 protein expression and total SOD activity in LECs (Vu *et al*., 2012) and in LSCs (Vu *et al*., 2013) were biphasically (it increased initially, then decreased) regulated by both PGF and H_2O_2. The present findings suggest that LSCs are targets of the luteolytic action of PGF and that PGF induces luteolysis by regulating antioxidant enzymes not only in LECs but also in LSCs. In the present study, PGF and H_2O_2 decreased 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 CAT and GPx decreased 24 h post-luteolytic PGF treatment. Surprisingly, CAT protein expression and CAT activity did not change while GPx1 protein expression and GPx activity significantly increased at 2 h in cultured LSCs treated with PGF and H_2O_2, indicating that PGF may differently regulate CAT and GPx. The reason for the transient increases in GPx after exposure of the cultured cells to PGF and H_2O_2 is unknown.

In conclusion, we demonstrated herewith that PGF and ROS regulate the expressions and activities of the antioxidant enzymes CAT and GPx, in the bovine CL, more specifically LSCs, suggesting that these enzymes are involved in the action mechanism of PGF in bovine CL. The down-regulation of these protein expressions and activities during structural luteolysis could enhance the accumulation of reactive oxygen species, which would result in both increasing luteal PGF production and cell death to complete CL regression in cattle.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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