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The Down-Regulation of Glutathione Peroxidase Causes Bovine Luteal Cell Apoptosis during Structural Luteolysis

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Prostaglandin (PG) $F_{2\alpha}$ is known to initiate luteal cell apoptosis in the bovine corpus luteum (CL) *via* its specific receptor (FP) on the luteal membrane by inducing intracellular Ca^{2+} mobilization and the activation of PKC. In order to identify the signaling components involved in cell apoptosis, mRNA levels and activities of antioxidative enzymes were analyzed using bovine CL at different stages of the estrous cycle. Northern blot analysis revealed that the levels of two isozymes of superoxide dismutase (SOD), the Mn and Cu/Zn types, and catalase are highly enriched in the middle estrous phase, whereas glutathione peroxidase (GPx) levels gradually decrease as the estrous cycle progresses. The incubation of bovine luteal cells with H_2O_2 and mercaptosuccinate (MS), a specific inhibitor of GPx, resulted in an increase in chromatin DNA condensation and apoptotic DNA fragmentation. Analyses of the enzymatic activities of GPx and catalase support the RNA data, indicating that H_2O_2 produced due to the lack of GPx is a potent inducer of luteal cell apoptosis.

Key words: apoptosis, estrous cycle, glutathione peroxidase, hydrogen peroxide, prostaglandin.

Progesterone (P4), a typical steroid hormone secreted from mammalian corpus luteum (CL), is essential for the implantation of blastocytes and the maintenance of pregnancy. If pregnancy is not established, the CL regresses in both a functional and structural manner in response to endometrial prostaglandin (PG) $F_{2\alpha}$ through its specific receptor (FP) on the luteal membrane (*1, 2*). During the regression phase of the estrous cycle, functional luteolysis associated with a decline in P4 levels occurs, followed by structural luteolysis accompanied by internucleosomal cleavage of genomic DNA, a characteristic of apoptosis (*3, 4*). This $PGF_{2\alpha}$-dependent luteolysis is indispensable for the ovulation of other follicles to control the cycling of normal estrous.

Reactive oxygen species (ROS), including superoxide, H_2O_2 and hydroxyl radicals, have been reported to be generated in rat (*5–7*) and bovine (*8*) CL during regression. ROS are produced by electron leakage from the mitochondrial P450scc system (*9, 10*) and cause irreversible damage to cellular DNA. Meanwhile, the activities of antioxidative enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), change drastically during the estrous cycle (*11*) and pregnancy (*12*). Previous reports have indicated that ROS induce the death of certain cells in the ovary, typically by follicle atresia and luteolysis (*13, 14*). These apoptotic events have been found to be suppressed efficiently by oestradiol (*15*), ascorbic acid and other antioxidants (*16*). It should be noted that $PGF_{2\alpha}$ induces ROS production in the CL *via* the activation of protein kinase C and intracellular Ca^{2+} mobilization (*5, 8*).

To identify the regulators of luteal-cell apoptosis, differential display and Northern blot analysis were carried out. In contrast to many antioxidative enzymes, the levels of GPx were found to decline in the CL just before the start of apoptotic events, suggesting that H_2O_2 facilitates luteal cell apoptosis in the bovine estrous cycle.

MATERIALS AND METHODS

Animals—CL-containing ovaries were collected at a local abattoir from Holstein cows or Japanese Blacks within 1 h after slaughter. The estrous stage of each CL was determined by morphological observation, as previously reported (*17*), and characterized as either early (3–5 days after ovulation), middle (8–12 days), late (15–18 days), or regressed (20–21 days). After classification of the luteal stage, CL were isolated and frozen in liquid nitrogen for the preparation of RNA. For the primary culture of luteal cells, middle- or late-phase CL were chilled in ice-cold saline (0.9% NaCl, 200,000 units/liter penicillin G potassium, 200 mg/liter streptomycin sulfate) until the start of the experiment.

Differential Display—Differential display of mRNAs from CL in early, middle, and regressed phases was performed under previously described conditions (*18*). In brief, reverse-transcribed cDNAs were subjected to polymerase chain reaction (PCR) amplification with 20 arbitirary and $T_{12}MN$ primers (M = A or G or C, N = A or G or C or T), followed by denaturing acrylamide-agarose gel electrophoresis. The detected DNA bands that were appeared specifically in each luteal stage, were extracted from the gel and

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Abbreviations: CL, corpus luteum; GPx, glutathione peroxidase; FP, prostaglandin $F_{2\alpha}$ receptor; MS, mercaptosuccinate; P4, progesterone; PG, prostaglandin; ROS, reactive oxygen species; SOD, superoxide dismutase.

reamplified by PCR. After subcloning into pUC19, the sequences were determined, and a BLAST search was carried out against all obtained samples.

Preparation of cDNA Probes—The partial cDNA (SN463) for bovine FP, which includes the IV through VI transmembrane regions (*19*), was used as a hybridization probe. Mn SOD cDNA was obtained by differential display analysis (see "RESULTS"). The cDNA fragments of bovine Cu/Zn SOD, catalase, and GPx were prepared by a standard RT-PCR method and used as hybridization probes. For the amplification of Cu/Zn SOD, sense (5'-CTAGAATTCATG-GCGACGAAGGCC-3') and antisense (5'-CTAGAATTCAG-TTCTCATTACAGG-3') primers were designed according to previously reported sequences (*20*). In addition, bovine GPx sense (5'-CTAGAATTCTGCTCTGGATTCGGAA-3') and antisense (5'-CTAGAATTCATGAGGAGCTGTGGTC-3') primers were synthesized based on previously published sequences (*21*). For the amplification of bovine catalase cDNA, PCR primers were designed based on porcine (*22*) and human (*23*) catalase as sense (5'-CTAGAATTCTGG-GACTTCTGGAGCC-3') and antisense (5'-CTAGAATTCG-GTGAGT GTCAGGAT-3') primers, respectively.

cDNA was synthesized with poly(A)$^+$ RNA prepared from early- and middle-phase CL by SuperScriptII (Life Technologies, Tokyo) reverse transcriptase. The PCR products were analyzed in 1.0% agarose gels and then subcloned into pUC19; each insert DNA was then identified by sequencing.

Northern Blot Analysis—Total RNAs were prepared from bovine CL using the guanidinium thiocyanate method as previously described (*24*). Fifteen micrograms of total RNAs were separated in 0.8% formaldehyde-agarose gels and transferred onto a Nytran nylon membrane filter (Schleicher & Schuell, Dassel, Germany). The blot was sequentially hybridized with FP, Mn SOD, Cu/Zn SOD, catalase, GPx, and GAPDH cDNA probes. The cDNA probes were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Uppsala, Sweden) by the *Bca*Best DNA labeling kit (Takara, Kyoto). Northern hybridization was performed under the following conditions: 50% formamide, 0.2% SDS, 5× SSPE [180 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate (pH 7.7)], 5× Denhardt's solution, and 100 μg/ml sonicated salmon testis DNA for 14 h. After the membrane was washed with 6× SSPE and 0.5% SDS solution, the filter was exposed to an imaging plate (IP, Fujifilm, Tokyo) for 6 to 12 h. IP scanning and intensity measurements of each hybridized band were performed by a Bio Imaging analyzer (BAS-2000, Fujifilm, Tokyo). A total of three independent hybridizations were performed, and average values were determined and are plotted in Fig. 2.

Catalase and GPx Assay—To determine the antioxidative enzyme activity, bovine CL at different stages were collected and chilled on ice with RIPA buffer [5 mM EDTA, 0.01% digitonin, 0.25% sodium cholate in PBS (–)]. The homogenate was centrifuged, and the supernatant was recovered for the following assays.

GPx activity was measured by a coupled enzyme assay (*25*). A 1 ml sample of reaction mixture (2 mM GSH, 1 U/ml glutathione reductase, 0.2 mM NADPH, 70 μM *t*-butyl hydroperoxide) and 10 μl of homogenated sample were mixed for assay. The decrease in the absorbance of NADPH at 340 nm was monitored. One unit of GPx activity was defined as the enzyme activity that consumes 1 μmol NADPH/min.

According to the standard method, catalase activity was measured by monitoring H_2O_2 metabolism at 240 nm. One unit of catalase activity was defined as the enzyme activity that decomposes 1 mM H_2O_2/min.

Preparation of Luteal Cells in Primary Culture—Bovine luteal cells in primary culture were prepared as described previously (*26*) with small modifications. Briefly, middle- or late-phase CL were sequentially perfused with EGTA buffer (10 mM HEPES, 140 mM NaCl, 7.1 mM KCl, 0.1 mM EGTA, pH 7.4), wash buffer (10 mM HEPES, 140 mM NaCl, 7.1 mM KCl, 5 mM $CaCl_2$, pH 7.4), and collagenase buffer (10 mM HEPES, 140 mM NaCl, 7.1 mM KCl, 5 mM $CaCl_2$, pH 7.4, 0.05% collagenase (Type I, Sigma, St. Louis, MO)). The collected luteal cells were washed three times with Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (D/F-12, Life Technologies, Tokyo) and grown in D/F-12 supplemented with 10% fetal bovine serum (FBS, Sanko Jyunyaku, Tokyo) and 20 μg/ml gentamicin (Life Technologies, Tokyo). Luteal cell viability was determined by trypan blue exclusion assay.

Extraction and Analysis of Fragmented DNA—The cultured luteal cells were treated with H_2O_2 and mercaptosuccinate (MS) or 3-amino-1,2,4-triazole (AT). After treatment for 1 to 4 h, genomic DNA was isolated from the luteal cells as follows. Luteal cells (5.0×10^5) were pelleted and suspended in lysis buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% sodium-*N*-lauroylsarcosinate), and then treated with RNase A and proteinase K. The DNA dissolving solution was extracted with phenol–chloroform and precipitated by ethanol. The recovered genomic DNA was separated by 1.5% agarose gel electorophoresis and visualized by ethidium-bromide (Et-Br) staining.

The isolated genomic DNA was end-labeled with [α-^{32}P]ddATP (Amersham Pharmacia Biotech, Uppsala, Sweden) by terminal deoxynucleotidyl transferase (TdT, Life Technologies, Tokyo) in a standard manner. Labeled DNA was analyzed by 1.5% agarose gel electorophoresis, followed by gel-drying and autoradiography.

Induction of Chromatin Condensation by H_2O_2 Treatment—Bovine luteal cells prepared from middle-phase CL were exposed to 10 μM H_2O_2 and 250 μM MS for 4 h, and then stained with 176 μM of Hoechst 33258. Cells that had undergone apoptosis, as indicated by fluorostained chromatin, were observed under a fluorescent microscope (Curl Zweiss, AXIOSKOP50).

RESULTS

Identification of Middle-Phase Enriched mRNA by Differential Display—In order to identify the signal mediator that induces luteal-cell apoptosis in luteolysis, mRNA derived from bovine CL in each estrous phase was analyzed by differential display analysis. Total RNAs isolated from early, middle, and regressed phases were subjected to differential display analysis as described in "MATERIALS AND METHODS." A total of 25 clones representing mRNA in each estrous phase were successfully isolated: two clones for the early phase, two for the middle, eleven for the early and middle, and ten for the regressed. BLAST search revealed that early and middle phase-specific clones correspond to bovine Mn SOD, consisting of a 378 bp 3' flanking region (+919 to +1296). One of the early phase-specific clones was identified as the α subunit of CD1 integrin (*27*), and a mid-

dle phase-specific clone corresponded to the X isoform of the human Dead box gene (*28*).

To ascertain the mRNA levels for Mn SOD and other proteins in CL, Northern blot analysis was employed using total RNA prepared from CL at different stages of the estrous cycle. Mn SOD levels increased markedly (a fivefold increase) from early to late phase in the estrous cycle, and then decreased during the regressed phase (Figs. 1A and 2A). Variations in the Mn SOD mRNA levels during the estrous cycle were consistent with the data from differential display analysis. Although the mRNA for the α subunit of CD1 integrin was markedly enriched at the early phase, only a low level of expression was observed for the dead box isoform (data not shown).

mRNA Expression of Cu/Zn SOD, Catalase, and GPx during the Estrous Cycle—The mRNA levels of other antioxidative enzymes, such as Cu/Zn SOD, catalase, and GPx, in the bovine CL were further investigated by Northern blot analysis. The levels of Cu/Zn-type SOD as well as Mn SOD increased significantly (a fourfold increase) from early to late phase, but then decreased at the regressed phase (Figs. 1B and 2B). Thus, sufficient amounts of both Mn and Cu/Zn SOD are generated in the luteal cells to protect the cells from radical attack during middle and late phases.

Subsequently, we also examined the mRNA expression of catalase and GPx. Similar to those of the SODs, catalase mRNA levels multiplied during the estrous cycle (a twofold increase from early to late phase) (Figs. 1C and 2C). In contrast, the levels of GPx mRNA decreased gradually throughout the estrous cycle (0.6 fold increase from early to late) (Figs. 1D and 2D). Although catalase and GPx are both involved in the reduction of H_2O_2, their intracellular localizations (*29*) and K_m values are quite different (*30*), suggesting that GPx is more active in luteal cells.

Northern blot analysis for FP showed a major 5.0-kb band and minor 3.0-kb bands, as shown in Fig. 1E. The

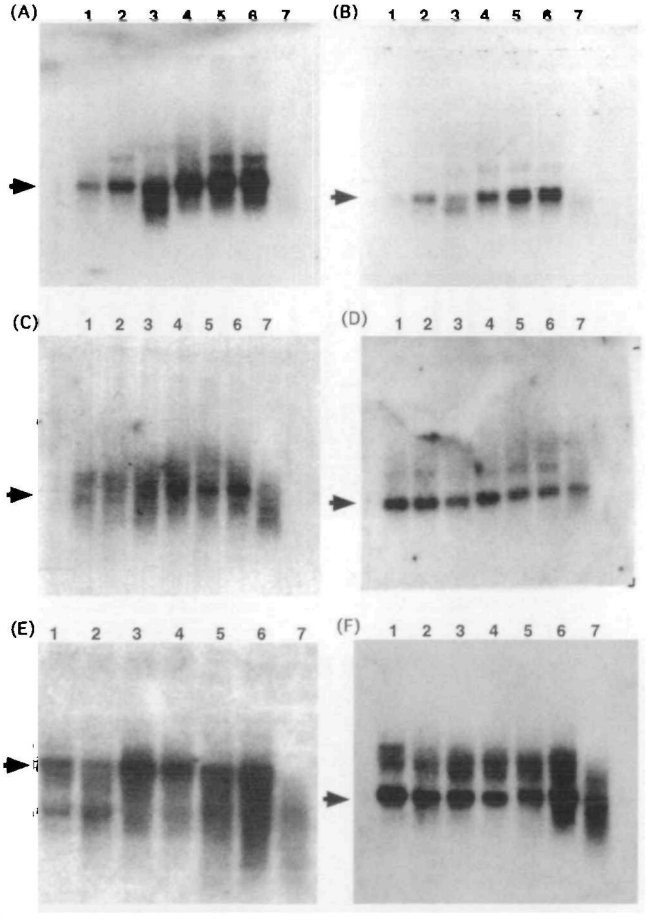

Fig. 1. **Analysis of mRNA levels of FP and antioxidative enzymes in bovine corpora lutea throughout the estrous cycle.** Total RNA was isolated from bovine corpora lutea at different stages of the estrous cycle (lanes 1 and 2, early; lanes 3–5, middle; lane 6, late; and lane 7, regressed stages) and transferred to a Nytran membrane for Northern analysis (see "MATERIALS AND METHODS"). The blot was sequentially probed with Mn SOD (A), Cu/Zn SOD (B), Catalase (C), GPx (D), FP (E), and GAPDH (F). A 1.4-kb Mn SOD mRNA, 1.6-kb Cu/Zn SOD mRNA, 2.2-kb catalase mRNA, 1.6-kb GPx mRNA, and 5.0-kb FP mRNA are indicated by arrowheads. Three independent experiments were performed using multiple CL samples ($n \geqq 3$) for each estrous stage. A typical hybridization blot is shown.

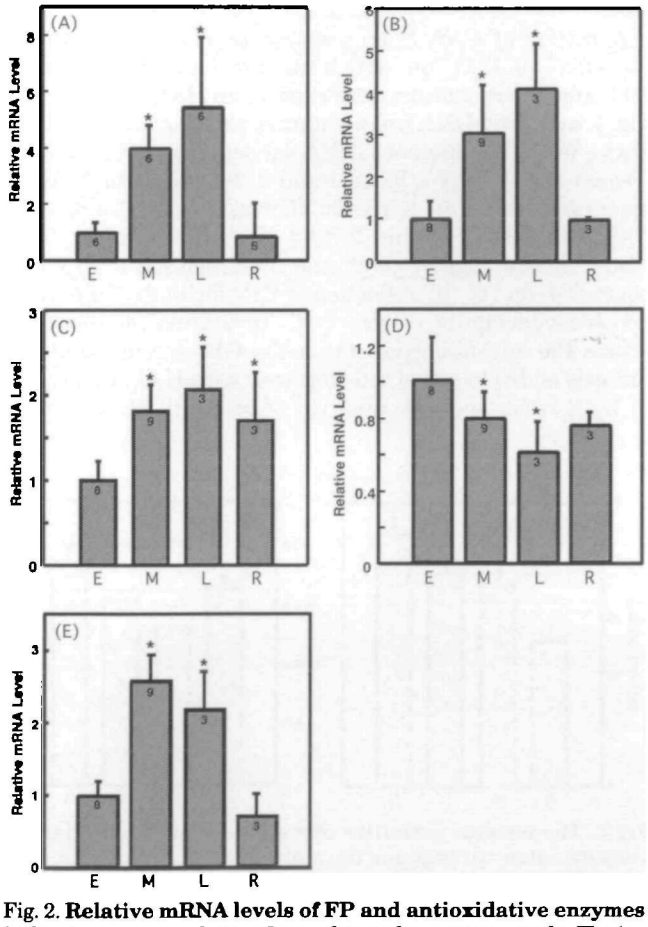

Fig. 2. **Relative mRNA levels of FP and antioxidative enzymes in bovine corpora lutea throughout the estrous cycle.** The intensities of the major bands for each mRNA [1.4-kb for Mn SOD (A), 1.6-kb for Cu/Zn SOD (B), 2.2-kb for catalase (C), 1.6-kb for GPx (D), and 5.0-kb for FP (E)] were measured by a BAS-2000 bioimage analyzer and normalized for GAPDH intensity. E, early stage; M, middle stage; L, late stage; and R, regressed stage. The number of samples used for Northern blot analysis are indicated in each column. Data from three independent experiments were combined and are shown as mean±S.D. of the relative intensity. (*) Results are statistically significant according to analysis of variance, with a p value of <0.05.

radioactivities of the major FP bands (5.0 kb) were measured and normalized against GAPDH radioactivity (Fig. 2E). The intensities of the 5.0-kb bands for FP mRNA increased from early to middle luteal phase (a two- to three-fold increase) and then dropped markedly at the end of the estrous cycle. These observations are consistent with previous data (*31*), suggesting that accumulation is essential for the initiation of the $PGF_{2\alpha}$ signal pathway and the subsequent Ca^{2+} influx and protein kinase C activation (*32*).

Activities of GPx and Catalase during the Estrous Cycle—In addition to mRNA analysis, the enzyme activities of catalase and GPx were determined using the procedures described in "MATERIALS AND METHODS." Consistent with the RNA data, catalase activity increased as the estrous cycle progressed (Fig. 3A), and GPx decreased from the early to the middle luteal phase (Fig. 3B). Because GPx is a prominent H_2O_2-metabolizing enzyme, a reduction in the GPx level should induce an accumulation of H_2O_2, which would lead to apoptosis of luteal cells downstream of the FP signaling pathway. In addition, it should be noted that functional luteolysis is induced by H_2O_2 in human CL (*33*).

Induction of DNA Fragmentation by H_2O_2—To evaluate the effect of H_2O_2 on structural luteolysis, bovine luteal cells in primary culture were exposed to H_2O_2. As shown in Fig. 4, apoptotic DNA fragmentation was induced by 10 μM H_2O_2. While spontaneous DNA ladders appeared in non-treated cells (Fig. 4A, lanes 1 and 2; B, lanes 1 to 4), H_2O_2 exposure caused an apparent increase in the amount of fragmented DNA within 2 to 4 h after treatment. This result clearly suggests that the accumulation of H_2O_2 in bovine CL due to the reduction of GPx facilitates luteal-cell apoptosis during the estrous cycle. To address whether GPx affects the anti-luteolytic status, the GPx-specific inhibitor MS was added to luteal cells together with H_2O_2. Compared to H_2O_2 treatment, the addition of MS with H_2O_2 further activated DNA fragmentation (Fig. 4A, lane 4; B and C, lanes 10 to 13), suggesting that GPx works as a potent anti-luteolytic agent in bovine luteal cells. Treatment with AT (3-amino-1,2,4-triazole), a specific catalase inhibitor, had no effect on DNA fragmentation (Fig. 4A, lane 5).

Induction of Chromatin Condensation by H_2O_2—In order to characterize the induction of cell apoptosis further, H_2O_2-treated luteal cells were stained with a fluorescence dye, Hoechst 33258, and the apoptotic cells were observed under a fluorescent microscope. The bovine luteal cells in primary culture were exposed to 10 μM H_2O_2 and 250 μM MS for 4

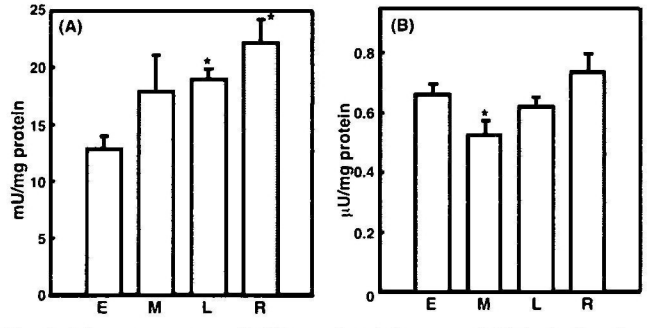

Fig. 3. **The enzyme activities of catalase and GPx in bovine corpora lutea throughout the estrous cycle.** For catalase and GPx assay, bovine corpora lutea were homogenized in RIPA buffer (5 mM EDTA, 0.01% digitonin, and 0.25% sodium cholate in PBS). The homogenate was centrifuged, and the supernatant was used for subsequent assays. Catalase activity was determined by following the decomposition of H_2O_2 at 240 nm (A); GPx activity was determined by following NADPH consumption at 340 nm (B) (see "MATERIALS AND METHODS"). E, early stage; M, middle stage; L, late stage; and R, regressed stage. One unit of catalase or GPx activity is defined as the amount reducing 1 mM H_2O_2/min or consuming 1 μmol NADPH/min, respectively. Experiments were performed independently in triplicate. The results are statistically significant according to analysis of variance, with a p value of <0.05 (*). Data are shown as the means±S.D. of each activity.

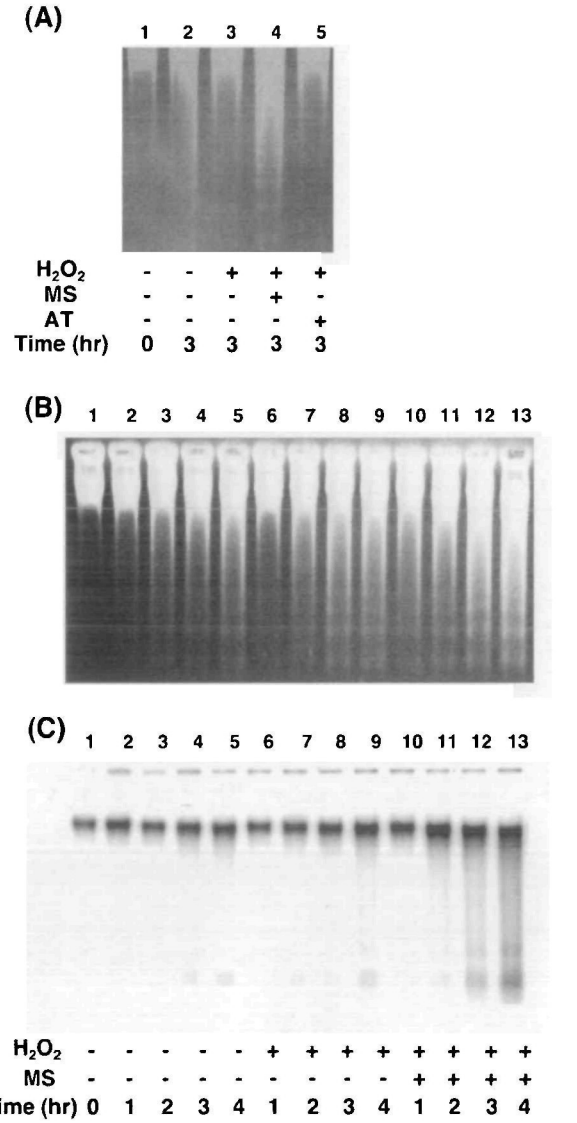

Fig. 4. **Induction of DNA fragmentation by H_2O_2.** Genomic DNA was isolated from bovine luteal cells in primary culture treated with H_2O_2, MS (mercaptosuccinate, a specific GPx inhibitor) and/or AT (3-amino-1,2,4-triazole). (A) Cells were treated for 3 h (no treatment, lane 2; 10 μM H_2O_2, lane 3; 10 μM H_2O_2 and 250 μM MS, lane 4; 10 μM H_2O_2 and 10 μM AT, lane 5) or 0 h (no treatment, lane 1). (B and C) Cells were treated with 10 μM MS for 0–4 h (no treatment, lanes 1–5; 10 μM H_2O_2, lanes 6–9; 10 μM H_2O_2 and 250 μM MS, lanes 10–13). Prepared DNA was separated in 1.5% agarose gels and stained by Et-Br (A and B). The DNA was end-labeled with [α-^{32}P]ddATP by TdT (C).

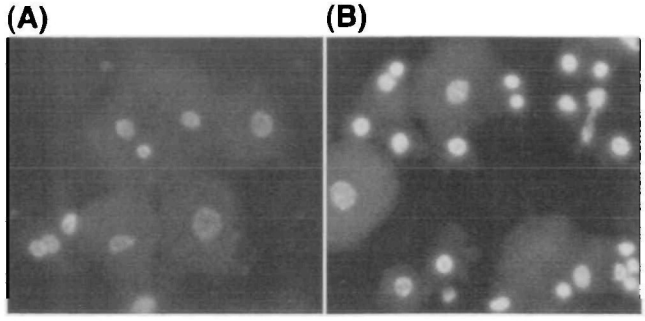

Fig. 5. **Induction of chromatin condensation by H_2O_2.** Bovine luteal cells prepared from middle estrous phase were treated with (B) or without (A) 10 μM H_2O_2 and 250 μM MS for 4 h. The cells were treated with 176 μM of Hoechst 33258, and stained nuclei were observed under a fluorescent microscope (AXIOSKOP50, Curl Zweiss).

h, and stained with 167 μM of Hoechst 33258. As indicated in Fig. 5, the bright nuclei accompanied by chromatin condensation were observed in H_2O_2-treated cells (Fig. 5B). The clusters of small cell bodies with fragmented nuclei indicate the production of typical apoptotic bodies.

DISCUSSION

It is well known that SOD converts superoxide to H_2O_2, thereby protecting cells from injurious radicals. Recently, several other groups have reported that oxygen radicals are produced during steroidogenesis by the P450scc system in mitochondria (*9, 10*).

In this report, we have clarified the mechanisms of structural luteolysis in the bovine ovary by observing the status of several antioxidative enzymes. Analyses of RNA expression and enzyme activities indicated that only GPx levels decrease throughout the estrous cycle, while the levels of other antioxidant emzymes increase, suggesting that GPx is a key mediator in the promotion of luteal cell apoptosis. The difference between the RNA level and enzyme activity of GPx (Figs. 2D and 3B) might be due to the existence of other GPx isozymes such as phospholipid hydroperoxide GPx. Because the most abundant GPx is the cytosolic type (*29*), which was analyzed in this study, cytosolic GPx could play a major role in protecting luteal cells from cell death. The cell lysate prepared without detergent showed a similar tendency to that shown in Fig. 3B, supporting the above idea (data not shown). A similar role for GPx has also been reported in other cells, including MDBK derived from bovine kidney (*34*). The catalase inhibitor AT, however, did not prevent DNA fragmentation in H_2O_2-treated cells (Fig. 4A, lane 5), indicating that GPx, not catalase, is the major scavenger of H_2O_2 in the bovine luteal cell. Eventually, the oxidative stress caused by H_2O_2 should affect both functional and structural luteolysis during the bovine estrous cycle.

An essential event in the induction of luteolysis is the activation of FP by its endogenous ligand, $PGF_{2\alpha}$. So far, FP has been well characterized in various animals, including bovines (*19*), mice (*35*), and humans (*36*), and has been revealed to be coupled to Gqα to form an effector protein (*37*). The importance of FP expression during luteal regression is strongly supported by a study of FP-deficient mouse in which functional luteolysis and parturition failed (*38*). Although the crucial role of FP has been demonstrated, researchers have not yet identified and characterized its downstream regulator. However, several lines of experimental evidence have demonstrated increases in the amounts of cyclooxygenase-2 (*39*) and endothelin-1 (*40*), as well as an induction of superoxide production (*5*) after $PGF_{2\alpha}$ treatment.

The results of the present study suggest that H_2O_2 accumulates due to a decrease in GPx, resulting in structural luteolysis. As the expression of bax and caspase-1 mRNAs increases in regressed CL (*41*), the involvement of oxidative stress in the transcription activities of such apoptosis-related genes remains an interesting subject. Moreover, it will be necessary to examine the association between FP stimulation and the expression of antioxidative enzymes, and to observe the localization of antioxidative enzymes in CL.

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