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Reactive Oxygen Species Up-Regulates Cyclooxygenase-2, p53, and Bax mRNA Expression in Bovine Luteal Cells

Tomohiro Nakamura and Kazuichi Sakamoto[1]
Institute of Biological Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan

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Reactive oxygen species (ROS) are well-established modulators of luteal cell apoptosis in the estrous cycle. The objective of this study was to clarify the molecular mechanisms of luteolysis by characterizing the levels and regions of mRNAs involved in ROS-induced luteal cell apoptosis. Stimulation of bovine luteal cells by H_2O_2 resulted in the induction of apoptotic nuclear condensation and Caspase-3 activation. In addition, a marker for oxidative stress-damaged DNA, 8-hydroxy-2′-deoxyguanosine, was highly accumulated in the large luteal cells prepared from the late estrous stage. Reverse transcription polymerase chain reaction and Northern blot analysis demonstrated that mRNAs of cyclooxygenase (COX)-2, p53, and Bax were highly accumulated in the H_2O_2-treated cells. *In situ* hybridization revealed that these mRNAs were most abundantly expressed in the large luteal cells. These findings suggest that enhancement of ROS in the bovine corpus luteum induces expression of COX-2, p53, and Bax mRNAs, resulting in activation of the signaling pathway for luteal-cell apoptosis.

Key Words: **cyclooxygenase; p53; Bax; prostaglandin; corpus luteum; 8-OHdG; H_2O_2 apoptosis.**

Programmed cell death is a highly conserved mechanism for removal of undesirable cells in vertebrate, and reactive oxygen species (ROS) play the regulatory roles in its signaling pathway. ROS are generated by the respiratory system of mitochondria (1, 2), and by the arachidonic acid cascade during prostaglandin (PG) production (3), and by P-450 isozymes implicated in steroidgenesis (4–6). Oxygen species are regulated by superoxide dismutase (SOD), an enzyme that changes superoxide into H_2O_2, or by catalase and glutathione peroxidase (GPx), which decompose H_2O_2 into H_2O. Because of their ability to degenerate ROS into nontoxic compounds, these antioxidative enzymes play a role in protecting cells from oxidative stress-induced cell death (7, 8).

When ROS overcomes the anti-apoptotic system, cells begin the process of suicide program. It is well established that up-regulation of the Fas/FasL system and release of cytochrome c are induced by H_2O_2 (9, 10). Cytochrome c binds to Apaf-1 and is an essential component for initiating the Caspase cascade via activation of Caspase-9 (11). Further nuclear translocation of p53 is known to be induced by H_2O_2 (12), and p53-induced apoptosis requires the generation of ROS (13) and transcriptional activation of Bax and Fas (14, 15).

Our group and others have already established ROS, and especially H_2O_2, as regulators of luteal-cell apoptosis during luteolysis (16–18). The bovine corpus luteum (CL) is maintained for almost 20 days and secretes progesterone to the mature endometrium in preparation for implantation of blastocysts. When pregnancy is not established, luteal regression is induced by uterine-derived $PGF_{2\alpha}$ (19). Therefore, elucidation of the molecular mechanisms underlying luteolysis is of critical importance for clarifying the process of parturition and pregnancy maintenance in vertebrates. To date, however, there have been few studies on the signaling mechanism of $PGF_{2\alpha}$-induced luteolysis. Recently, we found that glutathione peroxidase (GPx) was critically down-regulated during the development of CL (unpublished observation), suggesting that H_2O_2, a substrate of GPx, was accumulated in the late estrous stage and induced luteal cell apoptosis. Indeed, direct exposure of luteal cells to H_2O_2 *in vitro* have been shown to induce numerous apoptotic events, and thus it is proposed that ROS is a major activator of luteolysis.

The goal of the present study was to elucidate the induction mechanisms of luteal-cell apoptosis by ROS. For this purpose, we exposed primary cultured bovine luteal cells to H_2O_2, and identified and characterized the inducible mRNAs. The results showed that ROS up-regulated the mRNA expression of COX-2, p53, and Bax in the bovine CL, suggesting that these molecules are the critical effectors of luteal cell apoptosis.

[1] To whom correspondence should be addressed. Fax: +81-298-53-4676. E-mail: sakamoto@biol.tsukuba.ac.jp.

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MATERIALS AND METHODS

Animals and tissue preparations. For luteal cell culture, bovine (Holsteins or Japanese Blacks) ovaries with middle or late CL were isolated at a local abattoir and were kept in ice-cold saline. CLs were then staged based on morphological observations as early (3–5 days after ovulation), middle (8–12 days), late (15–18 days), or regressed (20–21 days) according to the method published previously (20). CLs were immediately excised, embedded in paraffin sections, and fixed in ice-cold 4% paraformaldehyde.

Cell culture. Luteal cells in primary culture were prepared as previously reported (21). The cells were maintained in Dulbecco's modified Eagle medium/Ham's nutrient mixture-12 (Life Technologies, Tokyo) supplemented with 5% FBS (Sanko Jyunyaku, Tokyo, Japan), 100,000 U/l penicillin G potassium, and 100 mg/l streptomycin sulfate. After culturing for 4 or 7 h, cells were harvested and fixed for Hoechst staining. Alternatively, after culturing for 3 or 6 h, cells were prepared for Western blotting, 8-hydroxy-2′-deoxyguanosine (8-OHdG) detection and RNA extraction.

Hoechst 33258 staining. Collected cells were fixed in 1% glutaraldehyde solution for 30 min then washed twice in PBS(−) and stained with 0.167 mM Hoechst 33258 (Sigma-Aldrich Japan, Tokyo). The fluo-stained nuclei were observed under a fluoromicroscope (Zeiss Axioscop 50; Zeiss Thomwood, NY) to detect the condensed nuclei, and apoptoted cells were counted.

Western blotting. The bovine luteal cells in primary culture were lysed by three rounds of freezing and thawing in lysis buffer (1% Triton-X, 50 mM Tris–HCl (pH 7.5), 2.5 mM EDTA, 250 mM NaCl). The cell lysates were separated on 12% SDS–PAGE and then electrotransferred onto a nitrocellulose membrane (Scheicher & Schuell, Dassel, Germany). Full-length and cleaved forms of caspase-3 were detected by anti-caspase-3 antibody (New England Biolabs, Beverly, MA) at a dilution of 1:1000. After the membrane was washed, the antibody was detected by LumiGLO chemiluminescent reagent (New England Biolabs, MA).

Immunohistochemical and immunocytochemical detection of 8-OHdG. Tissue specimens in paraffin blocks were cut into 8 μm sections and mounted on silane-coated slides. In order to detect the 8-OHdG levels quantitatively, sections from different CL stages were mounted on the same glass slides. Monoclonal anti-8-OHdG antibody (1:100; NOF Corporation, Tokyo, Japan) was incubated with the sections overnight at 4°C. Visualization of antibodies was performed with Dako LSAB2 Kit/HRP (Dako Japan, Kyoto, Japan) according to the manufacturer's instructions. In brief, the tissues were sequentially treated with biotinylated secondary antibody for 30 min, peroxidase-labeled streptoavidin for 10 min and 3-amino-9-ethylcarbazole solution for 30 min. To confirm the specificity of the primary antibody, a sample without the primary antibody was prepared as a negative control (Fig. 2f). The cells treated with 10 μM H_2O_2 and 250 μM MS were harvested and fixed for 8-OHdG detection. The cells were spotted onto glasses, and the 8-OHdG levels were immunocytochemically assessed as described above.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. The standard acid guanidinium method was used to extract total RNA from primary cultured luteal cells. Two micrograms of total RNA was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (MMLV; Sawady Tech., Tokyo, Japan) and oligo-dT primer. After addition of a 2 × PCR mix (Sawady Tech., Tokyo), PCR was then carried out for 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C. The following primer pairs were used for amplification: bovine COX-1 (sense, 5′-CTAGAATTCCAACCTTATCCCCAG-3′; antisense, 5′-CTAGAATTCATGGCGATGCGGTTGC-3′), COX-2 (sense, 5′-CTAGAA-TTCCAGATCACATTTGATTG-3′; antisense, 5′-CTAGAATTCT-GACTGTGGGAGGA-TAC-3′), FP (sense, 5′-ACTAGCGGCCGCGAAT-TCGGTGCTTGTTTGCTGAGTTA-3′; antisense, 5′-AACTAGTCGACG-CGGCCGCGTCAGCAGCACACACA-3′), liver-type PGF synthase (sense, 5′-GAGTGAAGCTCTGGAAGT-3′; antisense, 5′-GATCCCA-GAGCAC-CATAG-3′), lung-type PGF synthase (sense, 5′-GATCCCAAAAGT-CAGA-GG-3′; antisense, 5′-GCAGCATAAGCAACTAGG-3′), Mn-SOD (sense, 5′-AGATAT-GCAGCTGCACC-3′; antisense, 5′-CGTGTATCG-TGCAGTTAC-3′), CuZn-SOD (sense, 5′-GAATTCATGGCGACGAAG-GCC-3′; antisense, 5′-CTAGAATTCAGTTCTCATTA-CAGG-3′), catalase (sense, 5′-CTAGAATTCTGGGACTTCTGGAGCC-3′; antisense, 5′-CTAGAATTCGGTGAGTGTCAGGAT-3′), GPx (sense, 5′-CTAGAA-TTCTGCTCT-GGATTCGGAA-3′; antisense, 5′-CTAGAATTCATGAG-GAGCTGTGGTC-3′), p53 (sense, 5′-GGAGTATTTGGACGACCG-3′; antisense, 5′-TCAGTCTGAGTCAGGCCC-3′), Bax (sense, 5′-ATG-GACGGGTCCGGGGAG-3′; antisense, 5′-TCAGCCCATCTT-CTTCCA-3′), and GAPDH (sense, 5′-GACCCCTTCATTGACCT-3′; anti-sense, 5′-CCACCACCCTGTTGCTGT-3′). The sizes of the amplicons were as follows: COX-1, 792 bp; COX-2, 460 bp; FP, 424 bp; lung-type PGFS, 652 bp; liver-type PGFS, 572 bp; Mn-SOD, 523 bp; CuZn-SOD, 629 bp; catalase, 559 bp; GPx, 837 bp; p53, 574 bp; Bax, 579 bp, and GAPDH, 874 bp. PCR products were then separated on 1.0% agarose gel and visualized by ethidium-bromide staining.

Northern blot analysis. Total RNAs were prepared by the same method used for RT-PCR analysis. The RNA (10 μg) was separated by electrophoresis on 0.8% formaldehyde-agarose gel and transferred onto a nylon membrane (Scheicher & Schuell, Dassel, Germany). Hybridization was performed with ^{32}P-labeled cDNA fragments specific for bovine COX-2, p53, and Bax at 45°C in 5 × SSPE (1 × SSPE is composed of 0.18 M NaCl, 10 mM $NaPO_4$ (pH 7.7), and 1 mM EDTA), 2.5 × Denhardt's solution, 0.2% SDS and 100 μg/ml sonicated salmon sperm DNA. The cDNA fragments for the coding regions of +313 to +757 for COX-2, of +588 to +1161 for p53, and of +1 to +579 for Bax were used as probes for hybridization. After hybridization, filters were washed in 6 × SSPE-0.5% SDS, and signals were detected by BAS-5000 (Fujifilm, Tokyo, Japan). The filters were rehybridized with a cDNA fragment specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In situ hybridization. In situ hybridization was carried out as described previously (22). Paraffin sections of bovine CL were prepared as described above for immunohistochemical detection of 8-OHdG. The anti-sense and sense probes were synthesized by *in vitro* transcription in the presence of digoxigenin-labeled UTP (Roche Diagnostics, Mannheim, Germany). After penetration and hydration, the sections were fixed with 4% paraformaldehyde, acetylated with 0.25% acetic anhydride, and digested with 25 μg/ml proteinase K (Merck & CO., Whitehouse Station, NJ). Hybridization was carried out in a buffer containing 50% formamide, 2 × SSC, 1 mg/ml yeast tRNA, 1 mg/ml sonicated salmon sperm DNA, 1 mg/ml bovine serum albumin, and 10% dextran sulfate with incubation at 60°C. The slides were washed for 1 h in 2 × SSC, followed by treatment with 20 μg/ml ribonuclease A, and an additional wash in 0.1 × SSC for 30 min. The hybridized signals were detected using a DIG nucleic acid detection kit (Roche Diagnostics). Color development was performed by incubation with 4-nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution for 0.5 to 12 h.

RESULTS

ROS-induced luteal cell apoptosis. In our previous work, down-regulation of GPx level in the bovine CL during the estrous cycle resulted in accumulation of H_2O_2 and induction of luteal cell apoptosis (unpublished observation). In this report, we further characterized the mRNA expression of the genes involved in H_2O_2-induced apoptosis. When primary cultured luteal cells were exposed to H_2O_2 with GPx specific inhibitor (mercaptosuccinate: MS) for 4 h, apoptotic nuclear condensation was clearly observed (Fig. 1b), but no such

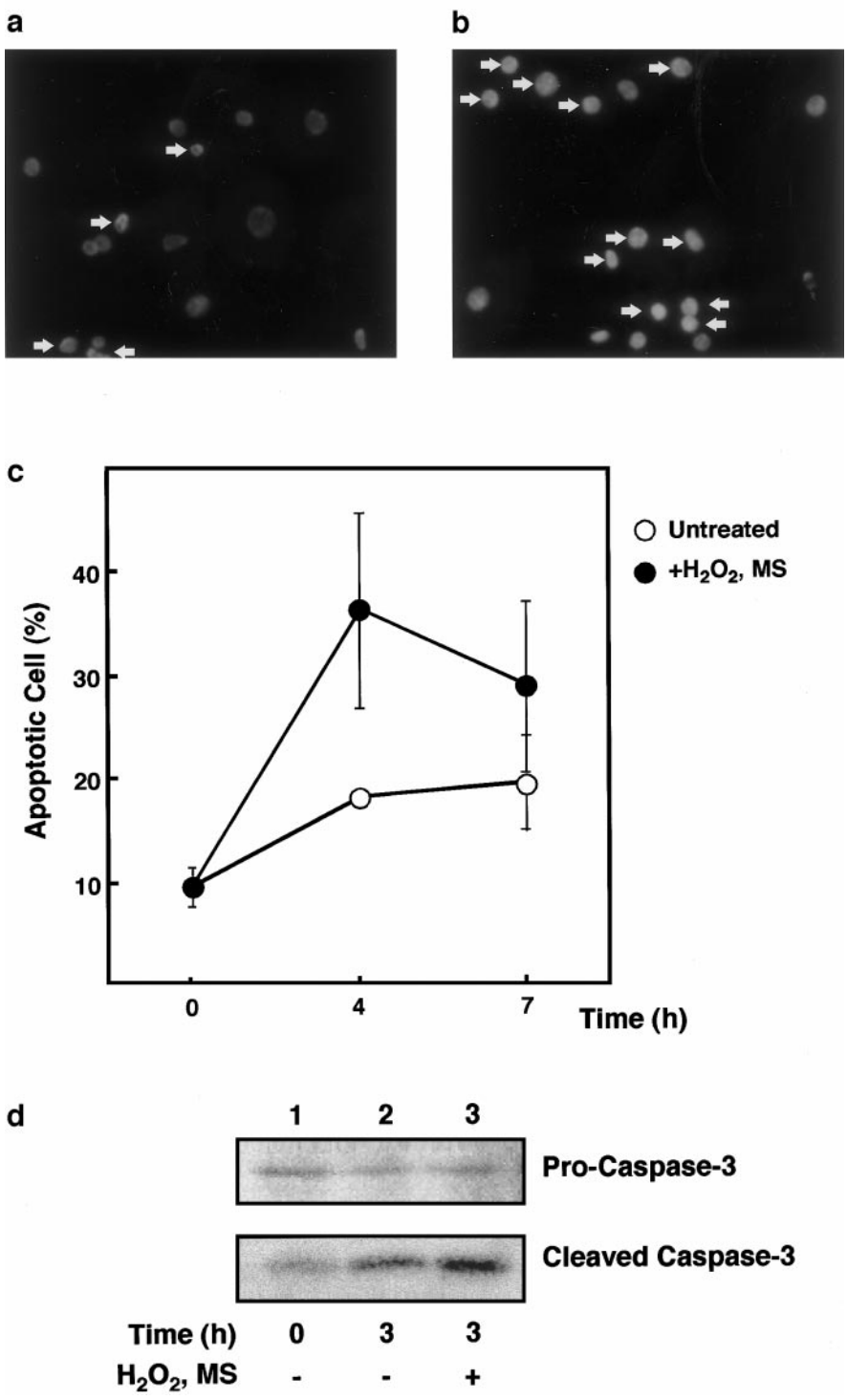

FIG. 1. Induction of luteal cell apoptosis by H_2O_2 and MS. Nuclear condensation was assessed using Hoechst 33258 for luteal cells in primary culture in the (a) absence or (b) presence of 10 μM H_2O_2 and 250 μM mercaptosuccinate (MS: a specific inhibitor of GPx) for 4 h. The arrows in panel (b) indicate condensed nuclei in the luteal cells. (c) The percentage of the cells undergoing apoptosis were determined by counting the condensed nuclei. Open circles indicate untreated cells and filled circles indicate ROS-treated cells. Error bars represent the standard deviation of three independent experiments. (d) Cleavage of caspase-3 into active form was detected by Western blot analysis. The cell lysates were extracted from the cells treated without (lanes 1 and 2) or with (lane 3) 10 μM H_2O_2 and 250 μM MS incubation for 0 h (lane 1) or 3 h (lanes 2 and 3).

condensation occurred in untreated cells (Fig. 1a). The number of condensed nuclei was twice that in untreated cells (Fig. 1c). These data strongly suggest that ROS is involved in the activation of luteal cell apoptosis. It is known that pro-caspase-3 is cleaved into smaller fragments by activated apoptotic signals, and that its cleaved form then activates CAD to complete DNA fragmentation (23). In the present study, the active form of caspase-3 was more abundantly detected in H_2O_2 and MS-treated luteal cells than in untreated cells (Fig. 1d, lanes 2 and 3). A cell extract from untreated cells, however, contained some amount of cleaved caspase-3, indicating that caspase-3 was naturally processed in the primary cultured luteal cells. Judging from these data, ROS-induced luteal cell death would appear to be dependent on caspase-3.

ROS-induced DNA damage in luteal cell. The amount of cellular DNA undergoing oxidative damage during the estrous cycle was assessed using antibody against 8-hydroxy-2′-deoxyguanosine (8-OHdG). The oxidative radicals preferentially attack cellular DNA, leading to DNA breakage and base modification, and these effects can be accurately measured by the accumulation of 8-OHdG levels (24). In this study, 8-OHdG abundance was assessed by means of an immunohistochemical procedure. Because luteal cell death *in vivo* occurs in the late estrous stage, we investigated whether the ROS was also generated at this stage. The sections of CL prepared from different estrous stages were used for immunohistochemistry. The specific binding of 8-OHdG antibodies was found only in the late CL, and signals were localized in the large luteal cells (Figs. 2c and 2d). The signal was not detected in the early, middle, or regressed stage (Figs. 2a, 2b, and 2e). The late-stage section reacted without antibody served as a negative control (Fig. 2f). Some middle CL, however, were stained weakly (data not shown), indicating that 8-OHdG is gradually accumulated in the CL from the middle to late stage. To estimate the amount of 8-OHdG more quantitatively, genomic DNAs prepared from CL in different stages were blotted onto a nitrocellulose filter, and exposed to the antibody, with the result that middle and late genomic DNA were shown to contain 8-OHdG (data not shown). Analysis of 8-OHdG levels revealed that ROS is most abundant in the late-stage CL, supporting our previous hypothesis (16–18) that ROS in the late stage augments luteal-cell apoptosis. Moreover, accumulation of 8-OHdG was observed in H_2O_2- and MS-treated luteal cells that were prepared from CL at the middle estrous stage (Fig. 2h), but no 8-OHdG accumulation was seen in untreated cells (Fig. 2g). This observation strongly indicates that an increase of H_2O_2 through deregulation of GPx causes generation of 8-OHdG in the large luteal cells and induction of luteal cell apoptosis.

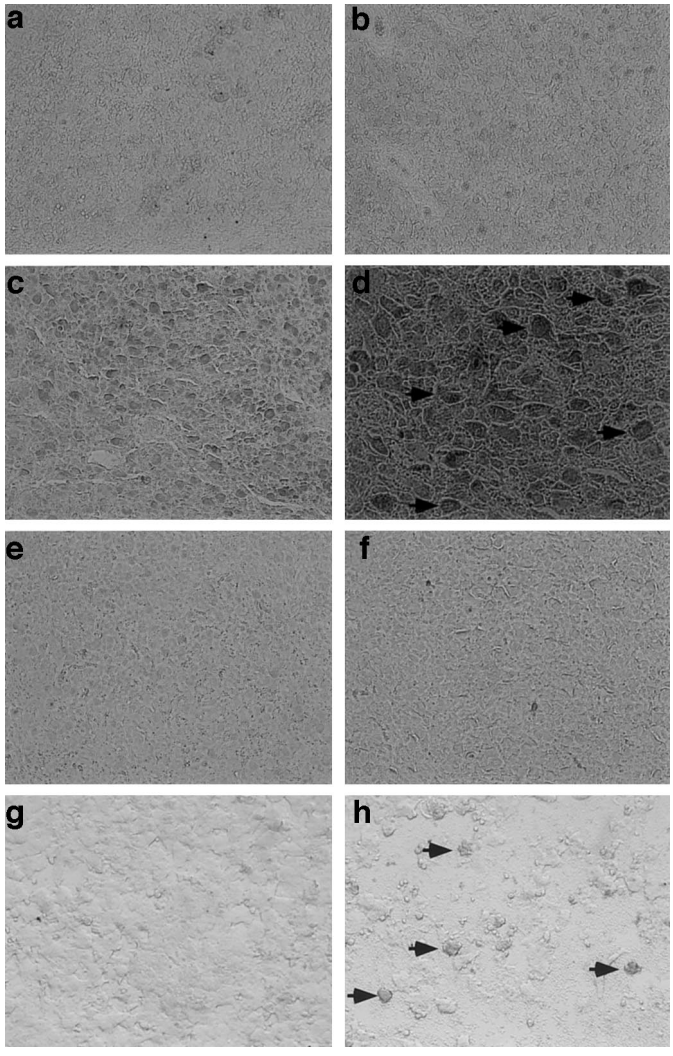

FIG. 2. Accumulation of oxidative stress in the cycling CL. The 8-hydroxy-2′-deoxyguanosine (8-OHdG: an oxidized DNA product) was immuno-histochemically detected in the bovine CL of (a) early, (b) middle, (c and d) late, and (e) regressed stages. Magnification of photomicroscopy in (a–c, e, and f) is 100×, and in (d, g, and h) is 200×. (f) Immunohistochemistry of a late-stage CL section without anti-8-OHdG antibody (negative control). Immunocytochemistry of 8-OHdG was performed with primary cultured-luteal cells that were (h) treated with 10 μM H_2O_2 and 250 μM MS, or (g) untreated. Arrows indicate the stained cells.

Screening and characterization of ROS-inducible mRNAs. Although uterine $PGF_{2\alpha}$ a potential initiator of luteal-cell apoptosis, and ROS are essential for induction of luteal regression, downstream effectors of these elements were almost completely unknown. We were therefore interested in the molecular mechanisms of the signaling pathway for ROS-activated luteolysis. To identify mRNA expression patterns in primary cells after ROS stimulation, RT-PCR analysis was carried out, and up-regulated-mRNAs were collected. A series of mRNAs considered to modulate luteal regression was selected and assayed. At first, PG-related factors

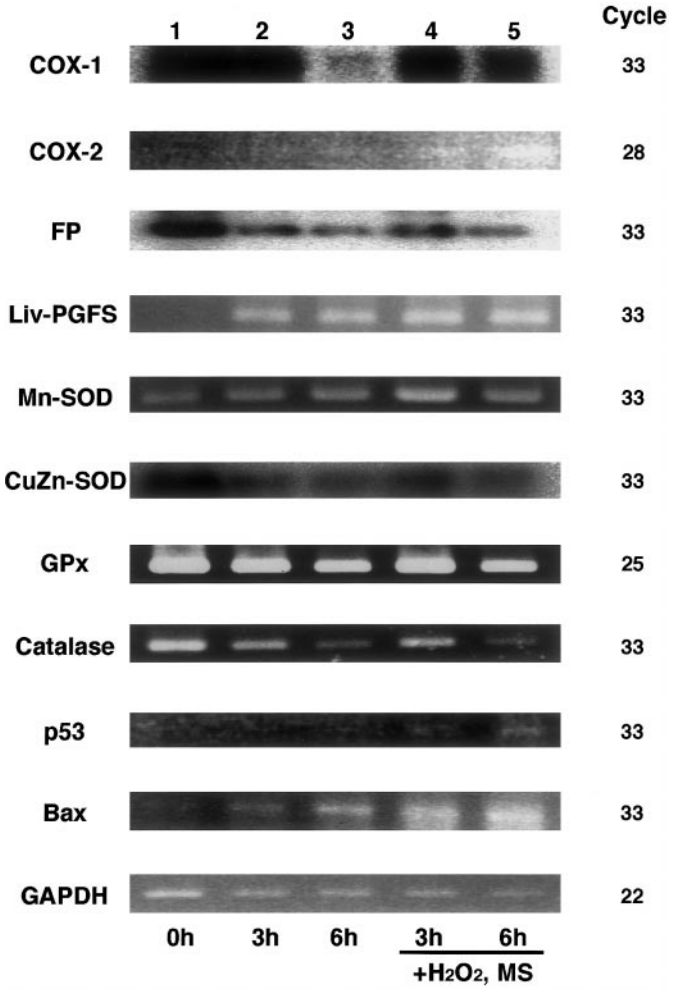

FIG. 3. mRNA expression of ROS-responsive genes in the bovine luteal cells. mRNA expression levels of COX-1, COX-2, FP, liver-type PGFS (Liv-PGFS), Mn-SOD, CuZn-SOD, GPx, catalase, p53, Bax, and GAPDH were determined by the standard RT-PCR method. Total RNAs were prepared from the cells cultured without (lanes 1, 2, and 3) or with (lanes 4 and 5) 10 μM H_2O_2 and 250 μM MS for 0 h (lane 1), 3 h (lanes 2 and 4) or 6 h (lanes 3 and 5). Because the mRNA levels of COX-1, FP, and CuZn-SOD were very low, each PCR-amplified band was detected by Southern blot analysis. The numbers shown at the right of the panel indicate the number of PCR cycles.

such as COX-1, COX-2, prostaglandin $F_{2\alpha}$ receptor (FP), liver-type PGF synthase (PGFS), and lung-type PGFS were analyzed. COX contains two isozymes, COX-1, and COX-2, and is the rate-limiting enzyme in the biosynthetic pathway of various PGs from arachidonic acid. It is generally understood that COX-2 is expressed inducibly by various biofactors (25, 26) and that COX-1 is constitutively expressed. Our results indicated that only COX-2 mRNA was increased in the cells cultured for 3 h after H_2O_2 and MS treatment (Fig. 3, lanes 2 and 4). The expression patterns, both COX-1 mRNA and FP mRNA, were similar between ROS-treated and -untreated cells (Fig. 3). Two isozymes of PGFS, the liver- and lung-type isozymes have been well characterized as the isomerases that change PGH_2 into $PGF_{2\alpha}$ (27, 28). In the present study, liver-type PGFS (Liv-PGFS) was abundantly expressed in the luteal cells, and was slightly increased by the ROS stimulus (Fig. 3, lanes 2 to 5), whereas lung-type PGFS was not detectable (data not shown), indicating that the liver-type is the major PGFS in the bovine CL.

For the next RT-PCR analysis, antioxidative enzymes of Mn-SOD, CuZn-SOD, catalase, and GPx were selected. These enzymes have been implicated in protecting the CL from luteolysis via reduction of the attack by oxidative radicals. RT-PCR analysis revealed that there was no significant change in RNA level between ROS-treated and -untreated cells (Fig. 3, lanes 1 to 5). Because the involvement of p53 and Bax in ROS-involved apoptosis has been well demonstrated (12–14), their expressions in the CL were also tested. As shown in Fig. 3, both p53 and Bax mRNA, especially the latter, were drastically up-regulated by stimulation with H_2O_2 and MS (lanes 4 and 5).

The mRNA expression of COX-2, p53, and Bax, which were identified as ROS-inducible genes in the bovine CL, was further characterized by Northern blot analysis (Fig. 4). Each of the single bands corresponding to COX-2, p53, and Bax mRNA was increased in ROS-stimulated cells at 3 and 6 h after incubation (Fig. 4, lanes 4 and 5), whereas GAPDH was stably expressed throughout the incubation (Fig. 4, lanes 1 to 5).

In situ hybridization for ROS-inducible mRNAs. To examine the cellular distribution of COX-2, p53, and Bax mRNA in the bovine CL, *in situ* hybridization was performed. Because the maximal level of oxidative stress was increased in the late-stage CL (Figs. 2c and 2d), the luteal sections obtained from the late estrous stage were used for *in situ* hybridization. For COX-2 and p53, strong hybridization signals for mRNA were

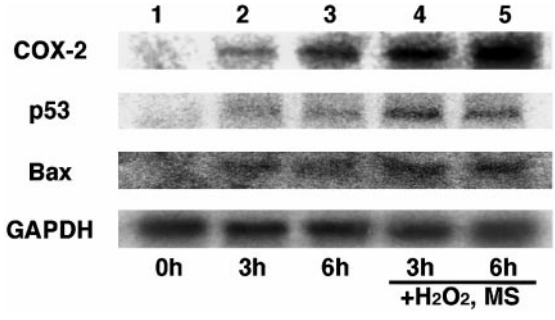

FIG. 4. Induction of COX-2, p53, and Bax mRNA expression by oxidative stress. Abundance of COX-2, p53, Bax, and GAPDH mRNA was quantified by Northern blot analysis. 10 μg of total RNAs from primary cultured luteal cells were separated on formaldehyde-agarose gel. The cells were cultured without (lanes 1, 2, and 3) or with (lanes 4 and 5) 10 μM H_2O_2 and 250 μM MS for 0 h (lane 1), 3 h (lanes 2 and 4), or 6 h (lanes 3 and 5). COX-2, p53, and Bax gave 4.1, 2.1, and 1.2-kb bands, respectively.

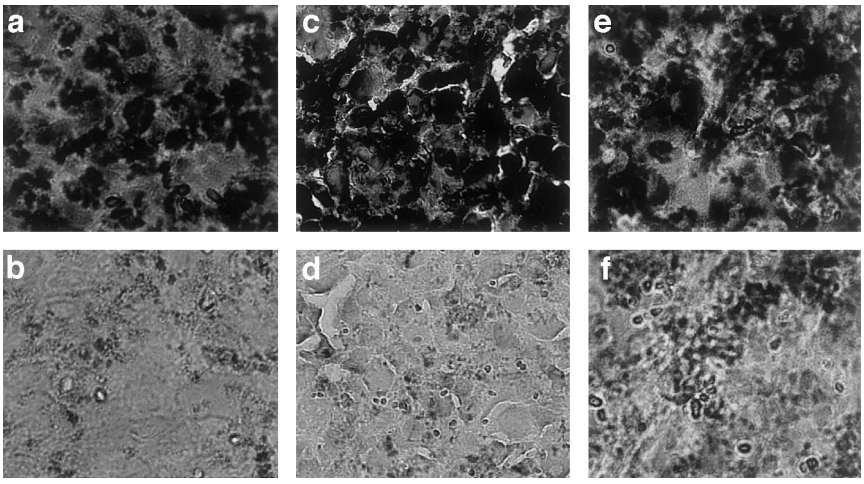

FIG. 5. Localization of COX-2, p53, and Bax mRNA in the bovine CL. *In situ* hybridization for the bovine CL with (a) COX-2 antisense, (b) COX-2 sense, (c) p53 antisense, (d) p53 sense, (e) Bax antisense, and (f) Bax sense probes. Paraffin sections were prepared from the late estrous stage and a photomicrograph was taken at a magnification of 400×.

observed, especially in the large luteal cells and hemocytes (Figs. 5a and 5c). Visualization of Bax mRNAs resulted in clear signals in the large luteal cells (Fig. 5e). No hybridization signals were detected when sense probes of COX-2, p53, and Bax were used as controls (Figs. 5b, 5d, and 5f). The findings in regard to 8-OHdG accumulation and specific mRNA induction in the same cell type (Figs. 2c and 2d; Figs. 5a, 5c and 5e) strongly suggest that oxidative stress induced COX-2, p53, and Bax expression in large luteal cells of the late estrous stage.

DISCUSSION

This study was the first to identify the ROS-activated genes in bovine luteal cells, which genes are known to be involved in oxidative stress-induced luteal cell apoptosis. Treatment with H_2O_2 and MS caused luteal cell apoptosis accompanied by nuclear condensation, caspase-3 activation, and mRNA accumulation of COX-2, p53, and Bax. Moreover, ROS production and expression of these mRNAs, were observed in the same cell type, indicating that ROS modulated luteolysis by regulating the expression levels of COX-2, p53, and Bax *in vivo*. The finding of Dharmarajan *et al.* reported that oxidative stress-induced luteal cell death is dependent on Bax expression (16) lends support to this hypothesis.

It has been well demonstrated that the most potent inducer of luteolysis is $PGF_{2\alpha}$, which stimulates ROS production as a byproduct of progesterone synthesis (5, 6). Recent studies have also shown that $PGF_{2\alpha}$ stimulates ROS generation via protein kinase C (PKC) in luteal cells (17, 29). Further, we previously demonstrated that the decline of GPx level during the estrous cycle caused the accumulation of H_2O_2 and structural luteolysis (unpublished observation). Although the signal interaction between $PGF_{2\alpha}$-activated PKC and GPx down-regulation is still unclear, the present study was the first to demonstrate that 8-OHdG was specifically enriched in the CL from the late estrous cycle, especially in the large luteal cells. Because FP is most enriched in large luteal cells (30), 8-OHdG accumulation in the same cell could be the result of steroid secretion induced by $PGF_{2\alpha}$. Colocalization of FP and ROS in the large luteal cells supports the idea that ROS mediate $PGF_{2\alpha}$-induced luteal cell apoptosis.

When ROS-induced apoptosis of luteal cells *in vitro*, typical nuclear condensation, and caspase-3 activation were observed. The effectors activated in the downstream of $PGF_{2\alpha}$ is little known; however, these findings suggest that the universal signaling pathway of apoptosis, namely, the release of cytochrome c, activation of various caspases, and DNA fragmentation by CAD would be underlying in the luteal cells (23). In addition, our data suggested that ROS activated the expression of COX-2, p53, and Bax mRNA, and these activations may be responsible for induction of luteolysis. Because the ubiquitous transcription factors, NF-κB and AP-1, can also be activated by oxidative stress (31–33), these factors could be involved in the transcriptional activation of COX-2 and p53 (34, 35) in bovine luteal cells. Expression of COX-2 resulted in the production of PGH_2, which would be further converted into $PGF_{2\alpha}$ by PGFS. The RT-PCR analysis in this study revealed that liver-type PGFS is the primary PGFS acting in the CL. In addition to the well-established initiation of luteolysis by uterine $PGF_{2\alpha}$, luteolysis may also be initiated by $PGF_{2\alpha}$ produced by the CL itself.

Expression of Bax and Fas is increased in cells undergoing p53-induced apoptosis (17, 18). The mRNA

expression of p53, however, is equivalent to that of Bax expression in ROS-treated luteal cells, and p53 may not be required for Bax expression in bovine luteal cells. In the mouse, SP1 is responsive for Bax expression, while p53-deficiency has no effect on Bax expression (36). This system may also be adapted to bovine luteal cells. However, the effect of ROS on phosphorylation of p53 and translocation of Bax into mitochondria remains to be clarified. Further studies will be needed to provide direct evidence of $PGF_{2\alpha}$ secretion in response to ROS.

In the present study, we found that ROS stimulation induced COX-2, p53, and Bax mRNA in the bovine CL, suggesting ROS contributes to luteolysis *in vivo.* More detailed investigations of this pathway will help our understanding of unsolved issues in mammalian pregnancy and parturition.

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