Prostaglandin (PG) $F_{2a}$ acting via a G protein-coupled receptor has been shown to induce apoptosis in the corpus luteum of many species. Studies were carried out to characterize changes in the apoptotic signaling cascade(s) culminating in luteal tissue apoptosis during PGF$_{2a}$-induced luteolysis in the bovine species, in which initiation of apoptosis was demonstrable at 18h after exogenous PGF$_{2a}$ treatment. Analysis of intrinsic arm of apoptotic signaling cascade elements revealed that PGF$_{2a}$ injection triggered increased ratio of Bax to Bcl-2 in the luteal tissue as early as 4h post treatment that remained elevated till 18h. This increase was associated with the elevation in the active caspase-9 and 3 protein levels and activity ($p < 0.05$) at 4-12h, but a spurt in the activity was seen only at 18h post treatment that could not be accounted for by the changes in the Bax/Bcl-2 ratio or changes in translocation of Bax to mitochondria. Examination of luteal tissue for FasL/Fas death receptor cascade revealed increased expression of FasL and Fas at 18h accompanied by a significant ($p < 0.05$) induction in the caspase-8 activity and tBid levels. Further, intrabursal administration of specific caspase inhibitors, downstream to the extrinsic and intrinsic apoptotic signaling cascades, in a pseudopregnant rat model revealed a greater importance of extrinsic apoptotic signaling cascade in mediating luteal tissue apoptosis during PGF$_{2a}$ treatment. The DNase responsible for PGF$_{2a}$-induced apoptotic DNA fragmentation was found to be a Ca$^{2+}$/Mg$^{2+}$ dependent, temperature sensitive DNase, optimally active at neutral pH conditions. This putative DNase was inhibited by the recombinant inhibitor of caspase activated DNase (CAD), and immunodepletion of CAD from luteal lysates abolished the observed DNA fragmentation activity. Together, these data demonstrate for the first time temporal and spatial changes in the apoptotic signaling cascades during PGF$_{2a}$-induced apoptosis in the corpus luteum.

Although PGF$_{2a}$ was discovered as a physiological luteolysin nearly three decades ago (1), cellular events associated with luteolysis remain poorly characterized, in part due to the lack of availability of a suitable in vitro model system that mimics all cellular events that occur in vivo in response to spontaneous or PGF$_{2a}$-induced luteolysis (2). Prostaglandin F$_{2a}$ interacts with its G protein-coupled receptor (GPCR), present predominantly on large luteal cells, but are also present on small luteal and endothelial cells of the corpus luteum (3) and activates Gq/PLC/PKC pathway (4, 5) resulting in decreased steroidogenesis, but intracellular signaling events that lead to structural regression of luteal tissue are poorly characterized. However, it is now well established that apoptosis or programmed cell death plays a central role in the structural regression of luteal tissue during PGF$_{2a}$-induced or spontaneous luteolysis of several species (6-10).

Apoptosis or programmed cell death is an evolutionarily conserved mechanism orchestrated by the host’s genome encoded proteins that form part of two distinct (intrinsic and extrinsic) signaling cascades. The intrinsic apoptotic signaling cascade is generally thought to be activated by apoptotic stimuli that originate within a cell in response to certain drugs, radiation, or growth factor withdrawal and primarily cause changes in mitochondrial permeability through alterations in the ratio of pro- to anti-apoptotic Bcl-2 family members (11). On the other hand, the extrinsic apoptotic signaling cascade is activated by extracellular signals (viz.,...
FasL) that interact with cell surface receptors (viz., Fas) to induce cell death (12). Changes in the mitochondrial permeability or death receptor activation lead to activation of a cascade of intracellular proteases known as caspases (13). Once activated, caspases cleave various cellular substrates including actin, poly (ADP-ribose) polymerase (PARP), DFF45/ICAD, fodrin and lamin that contribute to the morphological changes seen in apoptotic cells (13).

Fragmentation of DNA constitutes the final cellular event during apoptosis. It is mediated by the internucleosomal cleavage of DNA by endonucleases resulting in the formation of 180 bp DNA ladder, which is considered as one of the hallmarks of cellular apoptosis. Candidates for such endonucleases include the caspase activated enzymes such as DFF40/CAD (14, 15) and Nuc70 (16), divalent cation dependent neutral (17) or acidic endonucleases (18) and Ca++/Mg++ dependent endonucleases (19-21). Recently, however another DNase, Endo-G, released from mitochondria during apoptosis has also been shown to mediate apoptotic DNA fragmentation (22).

Unlike the classical cell surface receptors that induce cell death, PGF$_{2\alpha}$ receptor is a GPCR that lacks any intracytoplasmic region with an identity/ similarity to the death domain/ caspase activation and recruitment domain that recruits and/or activate caspases. However, caspase-8, one of the initiator caspases, acting downstream of the Fas receptor pathway has recently been reported to be activated during PGF$_{2\alpha}$-induced luteal tissue apoptosis in the corpus luteum of murine species (23) and the authors hypothesized that some level of cross-talk exists between PGF$_{2\alpha}$ receptor signal transduction and the classical apoptotic signaling cascades. This hypothesis is further strengthened by the observation that increased Bax and Fas expressions have been reported to be associated with the apoptosis in spontaneously regressing luteal tissue of the non-fertile cycles, while a significant attenuation in expression in these genes are seen in the corpus luteum during fertile cycle in the bovine species (24, 25). Also, several in vitro studies indicate that apoptotic stimuli such as, soluble FasL (25, 26), serum withdrawal (6), IFN gamma (27), and TNF-α (25, 28) that activate classical apoptotic signaling cascades are capable of inducing apoptosis in the luteal cells. The final phase of cellular demise i.e., fragmentation of DNA is very well characterized in the luteal tissue and is frequently used as an index of structural luteal regression. However, the nature of the DNase that executes DNA fragmentation during PGF$_{2\alpha}$-induced apoptosis in the luteal tissue has not been identified, though several DNase like activities viz., Ca++/Mg++ dependent DNase (29), Zn++ inhibitable DNase (6) and DNase I-like enzymes (30) have been observed to be active in the luteal cells under different conditions. These studies indicate that elements of apoptotic signaling cascades are present in the luteal cells, but the sequence of events that commit luteal cells to apoptosis in response to PGF$_{2\alpha}$ remains to be determined.

The purpose of the present study was to systematically analyze the involvement of classical apoptotic signaling cascades during PGF$_{2\alpha}$-mediated apoptotic cell death in the corpus luteum. Moreover, the biochemical nature of the endonuclease responsible for apoptotic DNA fragmentation in the corpus luteum in response to PGF$_{2\alpha}$ has not been previously studied and represents the major focus of our investigation. We chose to study these pathways in the corpus luteum of buffalo cows (Bubalus bubalis), since characterization of apoptosis following spontaneous or PGF$_{2\alpha}$-induced luteolysis in this species was recently reported (7). Our results show for the first time that PGF$_{2\alpha}$-mediated apoptosis in the corpus luteum involves activation of mitochondrial apoptotic signaling cascade that precedes the activation of FasL/Fas cascade during PGF$_{2\alpha}$-induced apoptotic cell death in the bovine corpus luteum. A pseudopregnant rat model was standardized to investigate the relative roles of intrinsic and extrinsic apoptotic signaling cascades in PGF$_{2\alpha}$-induced luteal tissue apoptosis. Using the pseudopregnant rat model, we demonstrate that intrabursal injection of an peptide based cell permeable caspase-8 inhibitor (Z-IETD-FMK) prior to PGF$_{2\alpha}$ injection abolished the caspase-3 activity and DNA fragmentation during PGF$_{2\alpha}$-induced luteal tissue apoptosis, whereas intrabursal injection of caspase-9 inhibitor (Z-LEHD-FMK) only attenuated the apoptosis. Moreover, our results point to an important role played by caspase activated DNase (CAD) in mediating apoptotic DNA fragmentation that follows with PGF$_{2\alpha}$-induced luteolysis.


**EXPERIMENTAL PROCEDURES**

**Reagents**

The polyclonal antibodies specific to Bcl-2 (# 197207), Bax (# 196820), phospho-BAD (# 9291), BAD (# 9292), cleaved caspase-9 (# 9501S), cleaved caspase-3 (# 9661S) Bid (# 2002) and ICAD (# PC366T) were purchased from Calbiochem-Novabiochem Corporation La Jolla, CA (# 197207, # 196820, # PC366T) and Cell Signaling Technology, Beverly, MA (# 9291, # 9292, # 9501S, # 9661S, # 2002). PARP antibody (AM30) was purchased from Oncogene Research Products, Boston MA. Caspase Activated DNase antibody (ab8406) was purchased from Abcam Inc., Cambridge, MA. β-Actin antibody (A5441) was purchased from Sigma-Aldrich Corporation, St. Louis, MO. Caspase substrates and inhibitors [Z-DEVD-AFC (Caspase-3 substrate # 264150), Z-IETD-AFC (Caspase-8 substrate # 368059), Z-LEHD-AFC (Caspase-9 substrate # 218765), Ac-DEVD-CHO (Caspase-3 inhibitor #235420), Z-IETD-FMK (Caspase-8 inhibitor # 218759), Z-LEHD-FMK (Caspase-9 inhibitor # 218761), Z-VAD(FM)K [(General executionary caspase (Caspase-1, 3, 4 and -7) inhibitor # 627610)] were purchased from Calbiochem-Novabiochem Corporation La Jolla, CA. In situ apoptosis analysis kit (#1684809) was purchased from Roche Diagnostics, Mannheim, Germany. The expression plasmid for glutathione S-transferase (GST)-ICAD-L (see reference 14 for details of the vector) was a kind gift from Professor S. Nagata, Department of Genetics, Osaka University Medical School, Osaka, Japan. Glutathione-Sepharose 4B and pGEX-5X3 (GST expression plasmid) were obtained from Amersham Pharmacia Biotech, Piscataway, NJ. Genescreen Plus and PVDF membranes were purchased from NEN Life Sciences, Boston, MA. All other reagents were purchased from Sigma-Aldrich Corporation, St. Louis, MO, Gibco BRL, Gaithersburg, MD, or sourced locally.

**Animal Models and Experimental Procedures**

**A. PGF$_2$α-Induced Luteal Tissue Apoptosis in the Buffalo Cows (Bubalus bubalis)**

All procedures in animals were approved by the Institutional Animal Ethics Committee, Indian Institute of Science. Buffalo cows (Bubalus bubalis) on day 11 of the estrous cycle were injected i.m., with 750 µg of Tiaprost (Iliren®, Intervet International B.V., Boxmeer, Holland) a synthetic analog of PGF$_2$α (n = 3-4 animals/time point). At 4, 12 and 18h following PGF$_2$α injection, ovaries were collected in cold PBS, and washed in PBS prior to processing. Under sterile conditions, the corpus luteum from the ovary was extirpated, cut into 6-8 pieces, transferred to labeled cryovials, flash frozen in liquid nitrogen and stored at -70°C until analysis. Also, a small portion of corpus luteum tissue was fixed in Bouin’s solution for histological examination.

**B. PGF$_2$α-Induced Luteal Tissue Apoptosis in the Pseudopregnant Rats: Effect of Caspase Inhibitors**

Adult rats (Wistar strain) were housed in a controlled environment (22-24°C) and kept under a photoperiod of 14h light and 10h of darkness with ad libitum access to food and water. Pseudopregnancy was induced in the female rats by cohabitation with vasectomized male rats on the afternoon of proestrus. Following cohabitation, female rats were examined for presence of vaginal plug and/or subjected to screening of vaginal smears daily for the extension of the diestrus period. Presence of vaginal plug/ day 1 of continuous diestrus (at least for 3 days) following cohabitation with the vasectomized male rats was considered as day 1 of pseudopregnancy. In addition, pseudopregnancy was also confirmed by serum progesterone analysis on day 5 of pseudopregnancy. Only the females showing serum progesterone >50 ng/ml were used in the study. On day 8 of pseudopregnancy female rats were anaesthetized with pentobarbital sodium (7.5 mg/rat, i.p.) and abdominal cavity was accessed through two dorsolateral subrenal incisions. Ovary with the surrounding tissues was exposed and a 30 gauge needle, attached to a 1 ml tuberculin syringe was gently entered into the ovarian bursa. Different caspase inhibitors (dissolved in DMSO) diluted in 0.15 M NaCl solution or vehicle solution for respective inhibitors (15 µg/ovary) were injected into the ovarian bursal cavity in a volume of 60 µl. Only animals with swelling of bursa and no visible leakage of the injected fluids were included in the study (n=3 rats/treatment group). The intrabursal route of administration has been utilized in various in vivo studies to deliver different drugs to the ovary (31-33). After the completion of the injection procedure, ovaries were returned to the abdominal cavity and the abdominal wall was sutured with absorbable suture and the skin with the surgical clips. Immediately after surgery (15 min), rats received...
(s.c.) either PGF$_{2\alpha}$ (15 µg Cloprostenol; Estrumat, Pitman-Moore Ltd., Harefield, UK) diluted in 0.3 ml 0.15 M NaCl or 0.3 ml 0.15 M NaCl (Vehicle) treatment. The animals were killed 24h later and the ovaries were visualized under a dissecting microscope and processed for corpora lutea collection. Corpora lutea were snap frozen in liquid nitrogen and stored at -70°C until analysis. The doses of peptide based cell permeable caspase inhibitors used in the present study were arrived at based on the previous studies where, as less as 300 ng of caspase inhibitors/rat have been found to effectively block apoptosis in several in vivo models of apoptosis, when injected directly into the tissue of interest (34-38). The luteolytic dose of PGF$_{2\alpha}$ employed in the present study was based on a previous study in the pseudopregnant rat model (39).

**In Situ Apoptosis Analysis, DNA fragmentation analysis and Immunohistochemistry**

Corpora lutea were processed for in situ apoptosis analysis according to the manufacturer’s recommendations. DNA fragmentation analysis and immunohistochemistry using Bax polyclonal antibody was carried out as described previously by us (7).

**RNA Extraction and Semiquantitative RT-PCR Analysis**

Total RNA was extracted from luteal tissue using Trizol reagent according to the manufacturer’s recommendations. The quality and quantity of each RNA sample was assessed spectrophotometrically and on a 1% formaldehyde agarose gel. Semiquantitative RT-PCR was carried out essentially as described previously (40). Oligonucleotide primers were designed for Bax, FasL, Fas and L-19 genes based on the conserved regions present in mRNA sequences of Homo sapiens, Bos taurus and Mus musculus. Primers (Forward and Reverse) and PCR conditions (annealing temperature and PCR cycle number) used were as follows: 5'-tcatcaggtgctagaggg-3' and 5'-cggccccgttggaatcc-3' for 237-bp Bax (56°C and 35 cycles); 5'-ccggctggtgactgtaacc-3' and 5'-ggagacagcggagaatca-3' for 128-bp Bcl-2 (53°C and 35 cycles) 5'-ataagtctcaccctccaacc-3' and 5'-cagccccattgctagccggg-3' for 174-bp FasL (55°C and 35 cycles); 5'-agggggaagcgctagcag-3' and 5'-gcaagggtcattgtttcac-3' for 178-bp Fas (50°C and 33 cycles); 5'-gaatcgccaatgccaacct-3' and 5'-tttagaccttagagctca-3' for 406-bp L-19 (58°C and 25 cycles). Ethidium bromide stained agarose gels displaying PCR products were scanned using UVI-Tech gel documentation system and quantitated using UVI-Band Map (1999) software.

**Western Blot Analysis**

Corpus luteum tissue lysate was prepared as per the previously published procedures (7). Equal amount of luteal tissue lysate (200 µg protein/lane) was resolved by 10% or 12% SDS-PAGE, electroblotted onto PVDF membrane and western blot analysis was performed as per the published procedures. Autoradiographs were scanned using UVI-Tech gel documentation system and quantitated using UVI-Band Map (1999) software.

**Caspase-8/9 Activity Assays**

Caspase-8/9 activity assays were performed using fluoroogenic substrates, Z-IETD-AFC (Caspase-8) and Z-LEHD-AFC (Caspase-9). In brief, luteal tissues collected before and after PGF$_{2\alpha}$ treatment, and after coinjection (15 min apart) of caspase inhibitors and PGF$_{2\alpha}$ were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM Na$_3$VO$_4$). After determining protein concentration an equal amount (200 µg) of luteal lysate protein (at non-saturating concentration of the assay, for details, see supplemental Fig. 1) was mixed in 0.5 ml of caspase-8/9 activity assay buffer (100 mM HEPES, pH 7.5, 20% Glycerol, 5 mM DTT and 0.5 mM EDTA) followed by the addition of 20 µM Z-IETD-AFC/10 µM Z-LEHD-AFC substrate. Reaction mixture was incubated at 37°C for 4-5h and liberated AFC was measured in a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength of 420-520 nm. Specificity of the caspase-8/9 assay was determined by the addition of caspase-8/9 specific inhibitor (Z-IETD-FMK/Z-LEHD-FMK).

**Caspase-3 Activity Assays**

Caspase-3 activity assays were performed using a fluorogetic substrate, Z-DEVD-AFC. Equal amounts (100 µg) of luteal lysate protein (at non-saturating concentration of the assay, for details, see supplemental Fig. 1) from different time points post PGF$_{2\alpha}$ treatment were added to 1 ml caspase-3 activity assay buffer (20 mM HEPES, pH 7.5, 10% Glycerol, 2 mM DTT) followed by the addition of 20 µM of Z-DEVD-AFC substrate. Reaction mixtures were...
incubated at 37°C for 1h and liberated AFC was measured in a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength of 420-520 nm. Specificity of the caspase-3 assay was determined by using caspase-3 specific inhibitor (Ac-DEVD-CHO).

Deoxyribonuclease Substrates

Rat liver nuclei were prepared as described previously (41, 42). In brief, rat liver was homogenized in homogenization buffer (15 mM HEPES-NaOH, pH 7.4, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine and 1 mM PMSF) containing 250 mM sucrose in a Dounce homogenizer. The homogenate was filtered through four layers of cheese cloth and an equal volume of homogenization buffer containing 2.3 M sucrose was added and mixed thoroughly. The homogenate was then layered over 5 ml of 2.3 M sucrose in a Beckman SW28 centrifuge tube and centrifuged at 22,000 x g for 90 min at 4°C. The pellet was resuspended in homogenization buffer at a concentration of ~3 x 10^6 nuclei/µl and stored at -70°C. Purified rat nuclei were used as a chromatin substrate for DNase activity assays. While the plasmid DNA obtained from pGEMT empty vector was used as a non-chromatin (naked DNA) substrate for DNase activity assays.

Deoxyribonuclease Activity Assays

For DNase assays crude cytosolic extracts were prepared from the luteal tissue essentially as described previously (43, 44). Briefly, luteal tissue collected before and at 18h post PGF2α treatment was swollen in an equal volume of extraction buffer (50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 20 µM cytochalasin B, 1 µg/ml leupeptin, 1 µg/ml pepstain and 1 mM PMSF) for 10 min followed by five cycles of freezing and thawing, which was accompanied by grinding with pestle each time. The resultant lysate was then centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was aspirated, aliquoted and frozen at -70°C till used for protein estimation by Bradford (microassay) method. This supernatant was used as crude luteal cytosolic extract for DNase assays.

DNase assays were performed according to the previously published procedures (43, 44) with some modifications. For DNase assay using non-chromatin substrate, 10 µg of plasmid DNA was incubated with 50 µg of crude luteal cytosolic extract at 37°C for 30 min (except for the time course analysis of DNase activity where assay was terminated at different time points) in a DNase assay buffer (20 mM HEPES, pH 7.5, 1 mM CaCl₂, 5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF if not stated otherwise). For analysis of pH dependence of the DNase activity, using naked DNA as a substrate, different buffers were used that included; Acetate-NaOH (pH 4.0 and 5.0), Phosphate buffer (pH 6.0), MOPS-NaOH (pH 6.5), HEPES-NaOH (pH 7.5) and Tris-HCl (pH 8.0 and 9.0) with the other components of the assay buffer remaining the same. Assay was terminated by the addition of 6X loading dye and samples were analyzed in a 2% agarose gel containing 0.5 µg/ml ethidium bromide.

For DNase assays using chromatin substrate, ~3 x 10^6 nuclei were incubated with crude luteal cytosolic extract in different pH assay buffers as indicated above for 30 min at 37°C. Assay was terminated by adding the lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2 M NaCl, 0.2% SDS and 0.1 mg/ml Proteinase-K) and DNA was precipitated after overnight incubation at 37°C by the addition of equal volume of Isopropanol. The resultant nucleic acid pellet was dissolved in Tris-HCl, pH 8.0 containing 1 mM EDTA and 0.1 mg/ml RNase A and incubated at 37°C for 30 min. The DNA was extracted and analyzed by gel electrophoresis using a 2% agarose gel in the presence of 0.5 µg/ml ethidium bromide.

Immunodepletion of Caspase Activated DNase

Immunoprecipitation was carried out by incubating 400 µg of regressing (18h after PGF2α treatment) luteal lysate protein with polyclonal anti-CAD antibody or rabbit IgG and Protein-A agarose at 4°C overnight. The pellet (P) containing immune complexes was obtained by centrifugation at 15,000 x g for 10 min at 4°C. The pellet was diluted in 50 µl of immunoprecipitation buffer and an aliquot was used in the in vitro DNase activity assay, whereas supernatant (S) was concentrated to a volume of 50 µl and then used in the DNase activity assay. Similar procedures were followed for control IgG supernatant and pellet.

Statistical Analyses

Data wherever applicable was expressed as mean ± SEM. The arbitrary densitometric units were represented as the percentage relative to control, which was set at 100%. The data were analyzed by one-way ANOVA followed by
RESULTS

Prostaglandin F$_{2\alpha}$ injection into Buffalo Cows Induces Luteal Cell Apoptosis in the Corpus Luteum

In situ apoptosis analysis of the luteal tissue collected at different time points after PGF$_{2\alpha}$ injection showed increased number of apoptotic cells (small and large luteal cells), 18h after PGF$_{2\alpha}$ injection into buffalo cows (Fig. 1B). While no incidence of apoptosis was observed in the corpus luteum at 4h, some evidence of apoptosis was present in the corpus luteum at 12h after PGF$_{2\alpha}$ injection. The present data confirms our previous observation of time of onset of apoptotic DNA fragmentation during PGF$_{2\alpha}$-induced luteolysis (7) and further validates our model for the analysis of apoptotic signaling cascades that culminate in the apoptotic cell death in the corpus luteum.

Activation of Mitochondrial Apoptotic Signaling Cascade in the Corpus Luteum during PGF$_{2\alpha}$-Induced Apoptosis

Figure 2 illustrates changes in steady state mRNA and protein levels of pro- and anti-apoptotic Bcl-2 family members during PGF$_{2\alpha}$-induced luteal tissue apoptosis. Bax (a pro-apoptotic gene) mRNA (Fig. 2A) and protein levels (Fig. 2B) increased within 4h and remained high at 18h, the last time point observed, but mRNA and protein levels of Bcl-2 (an anti-apoptotic gene) did not change in the luteal tissue post PGF$_{2\alpha}$ treatment (Fig. 2A and B). Changes in Bax and Bcl-2 mRNA and protein levels are also expressed as Bax/Bcl-2 ratio, an index that is used for the activation or inhibition of the mitochondrial permeability to apoptogenic molecules (Fig. 2C). As can be seen in Fig. 2C, Bax/Bcl-2 ratio, both mRNA and protein levels increased, with a more profound effect on the Bax/Bcl-2 protein ratio, as early as 4h, which remained high till 18h, suggesting an increase in the mitochondrial permeability to apoptogenic molecules. Immunohistochemical analysis of Bax (Fig. 2D) revealed increased punctate appearance of cells (in the perinuclear region) during PGF$_{2\alpha}$-induced luteal tissue apoptosis, a parameter that confirms increased localization of proteins to mitochondria (45). However, IgG control didn’t show any staining (Shown in Inset). Bad, another pro-apoptotic Bcl-2 family member that gets activated upon hypophosphorylation was also examined in the luteal tissue that showed decrease in the phosphorylation of Bad within 4h and the levels remained low at 18h (Supplemental Fig. 2A). Next, we determined changes in the active caspase-9 protein levels and activity during PGF$_{2\alpha}$-induced luteal tissue apoptosis. As seen in Fig. 3A, active caspase-9 protein levels increased in the luteal tissue 4-12h after PGF$_{2\alpha}$ treatment and was highest at 18h. In vitro caspase activity assays using caspase-9 specific substrate Z-LEHD-AFC essentially corroborated the western blot analysis results (Fig. 3B). Z-LEHD-FMK (a specific inhibitor of caspase-9) completely abolished the caspase activity observed in the luteal tissue lysates confirming the specificity of the assay (Fig. 3B).

Activation of Caspase-3 in the Corpus Luteum during PGF$_{2\alpha}$-Induced Apoptosis

To address whether caspase-9 activation (initiator caspase) was associated with increase in the activation of downstream target effector caspase; caspase-3, we measured active form of caspase-3 by western blot analysis and caspase-3 activity by in vitro caspase-3 activity assay. As shown in Fig. 4A, a dramatic increase in the active caspase-3 protein levels was seen at 18h. Increase in the active caspase-3 protein levels was associated with an increase in the cleaved form of PARP, an endogenous substrate of caspase-3, and a significant increase ($p < 0.05$) in the caspase-3 activity in the corpus luteum at 18h post PGF$_{2\alpha}$ treatment (Fig. 4B). Addition of Ac-DEVD-CHO (a specific inhibitor of caspase-3) completely abolished the caspase activity observed in the luteal tissue lysates confirming the specificity of the assay (Fig. 4B).

Analysis of Extrinsic Apoptotic Signaling Cascade Elements in the Corpus Luteum

Figure 5 illustrates changes in steady state mRNA levels of Fas and FasL in the corpus luteum before and at different time points after PGF$_{2\alpha}$ treatment. As can be seen in Fig. 5A, FasL mRNA expression levels were unchanged in the corpus luteum at 4 and 12h, but the expression was high at 18h. The Fas receptor mRNA levels were upregulated in the corpus luteum within 4h (4.5 fold compared to 0h), became low at 12h (1.4 fold compared to 0h), but increased again at 18h (2.3 fold compared to 0h). Analysis of caspase-8 activation revealed no change at 4-12h post...
treatment, but the activity increased significantly ($p < 0.05$) at 18h (Fig. 5C). Z-IETD-FMK (a specific inhibitor of caspase-8) completely abolished the caspase activity observed in the luteal tissue lysates confirming the specificity of the assay (Fig. 5C). Next, we determined changes in cleavage of Bid, a downstream target of caspase-8 that can interact with mitochondria and lead to amplification in the mitochondrial cascade. Immunoblot analysis of truncated Bid (tBid) levels in the corpus luteum is shown in Fig. 3B. While tBid was undetectable at 0, 4 and 12h, signal was high at 18h post PGF$_{2\alpha}$ treatment.

**Relative Contribution of Intrinsic and Extrinsic Apoptotic Signaling Cascades in PGF$_{2\alpha}$-Induced Apoptosis**

We next investigated relative contribution of intrinsic and extrinsic apoptotic signaling cascades in mediating PGF$_{2\alpha}$-induced luteal tissue apoptosis. For this purpose, we standardized a pseudopregnant rat model for PGF$_{2\alpha}$-induced luteolysis and employed pharmacological blockade of specific caspases associated with intrinsic and extrinsic apoptotic signaling cascades as well as general caspases, using peptide based cell permeable, irreversible caspase inhibitors. Before analyzing the effects of caspase inhibition on PGF$_{2\alpha}$-induced luteal tissue apoptosis in pseudopregnant rats, it was necessary to determine whether PGF$_{2\alpha}$ treatment activated similar apoptotic signaling cascades in the rat corpus luteum. The experimental design employed in rats is presented in Fig. 6A, and DNA fragmentation analysis, semiquantitative RT-PCR analysis of upstream activators of intrinsic and extrinsic apoptotic signaling cascades (Bax and FasL, respectively), and changes in caspase-9, 8 and 3 activities during PGF$_{2\alpha}$-induced luteal tissue apoptosis are presented in Fig. 6 B-F. As can be seen in Fig. 6B, increased DNA oligonucleosomes formation is discernible in the DNA isolated from luteal tissues collected 24h after PGF$_{2\alpha}$ injection, indicative of cell death through apoptosis. Semiquantitative RT-PCR analysis revealed upregulation in the steady state mRNA levels of Bax and FasL during PGF$_{2\alpha}$-induced apoptosis in the rat luteal tissue (Fig. 6C). We next determined whether increases in the expression of these genes that act upstream of apoptotic signaling cascades is associated with activation of downstream caspases, results of caspase activation analysis are presented in Fig. 6D-F. As can be seen from the Figures, a significant ($P<0.05$) upregulation in caspase-9 (1.9 ± 0.04 fold vs 0h), caspase-8 (3.5 ± 0.5 fold vs 0h) and caspase-3 (4.8 ± 0.3 fold vs 0h) activities occurred in the luteal tissue following PGF$_{2\alpha}$ injection. These results suggest striking similarities in the mechanisms of apoptosis initiation in the cow and rat luteal tissue and validate our choice of use of pseudopregnant rat model for examining the relative role of intrinsic and extrinsic apoptotic signaling cascades during PGF$_{2\alpha}$-induced luteal tissue apoptosis *in vivo*.

Figure 7A represents the experimental design utilized to examine the effect of peptide based cell permeable caspase inhibitors [Caspase-9 inhibitor (Z-LEHD-FMK), Caspase-8 inhibitor (Z-IETD-FMK) and general caspase inhibitor (Z-VAD-FMK)] on PGF$_{2\alpha}$-induced luteal tissue apoptosis and changes in caspase-3 activities and oligonucleosome formation in the luteal tissue are presented in Fig. 7B-D. Although caspase-9 inhibitor significantly ($P<0.05$) decreased PGF$_{2\alpha}$-induced caspase-3 activity, DNA oligonucleosome formation was only marginally decreased in the luteal tissue. In contrast, administration of caspase-8 inhibitor or general caspase inhibitor resulted in dramatic reduction in PGF$_{2\alpha}$-induced caspase-3 activity and oligonucleosome formation. These results suggest requirement of activation of extrinsic apoptotic signaling cascade, which is necessary and sufficient for PGF$_{2\alpha}$-induced apoptosis in the luteal tissue, whereas activation of intrinsic apoptotic signaling cascade alone is insufficient.

**Induction of a Ca$^{++}$/Mg$^{++}$ Dependent DNase Activity in Corpus Luteum during PGF$_{2\alpha}$-Induced Apoptosis**

In order to examine the DNase activity responsible for apoptotic DNA ladder formation during PGF$_{2\alpha}$-induced luteal tissue apoptosis, we analysed cellular DNase activity in the corpus luteum collected 18h post PGF$_{2\alpha}$. Before subjecting the PGF$_{2\alpha}$ treated tissues for characterization of DNase activity, tissues were analysed for DNA oligonucleosome formation (Supplemental Fig. 3) to confirm for the presence of DNase activity in corpus luteum collected from the buffalo cows 18h post PGF$_{2\alpha}$ injection. Biochemical characterization of the DNase activity in the crude cytosolic extract of corpus luteum collected 18h post PGF$_{2\alpha}$ treatment indicated that maximum DNase activity was observed under neutral pH conditions (pH 6.5-7.5)
(Fig. 8A). DNase activity was found to be dose-dependent, being maximum at 50 μg of cytosolic extract (Fig. 8B) and maximum activity was observed at 60 min (Fig. 8C) of the assay under the conditions tested. As shown in the Fig. 8D, the observed DNase activity was inhibited by the addition of EDTA/EGTA suggesting the requirement of divalent cations Ca"/Mg" by the DNase. Heat treatment inactivated the DNase activity, but RNase was ineffective suggesting proteinaceous nature of the DNase activity.

**Inhibition of DNase Activity by Inhibitor of Caspase Activated DNase (ICAD)**

Next, we examined changes in the ICAD fragmentation during PGF$_{2\alpha}$-induced luteal tissue apoptosis. Increased low molecular weight ICAD fragmentation products that corresponded to previously published (46, 47) partial ICAD fragments were observed following PGF$_{2\alpha}$-induced apoptosis in the corpus luteum (Supplemental Fig. 4). The chemical properties of DNase in association with the degradation of ICAD suggested that it could be caspase activated DNase (CAD). To examine this possibility, ICAD-L, a specific inhibitor of CAD was expressed in *E. coli* as a GST fusion protein and purified as described previously by Nagata and coworkers (14) (Supplemental Fig. 5). This GST-ICAD-L fusion protein was then used in an *in vitro* DNase assay to examine whether it can inhibit the DNase activity. As shown in Fig. 9, GST-ICAD-L was able to inhibit the DNase activity (oligonucleosome formation in nuclei based assay and DNA smear formation in plasmid DNA based assay) observed in the crude cytosolic extract prepared from the luteal tissue collected at 18h post PGF$_{2\alpha}$ treatment in a dose-dependent fashion. Complete inhibition was observed at a dose of 100 ng, while GST even at a concentration of 200 ng did not inhibit the DNase activity. GST-ICAD-L was not observed to inhibit DNase I or II activities (data not presented).

**Induction of Caspase Activated DNase in the Corpus Luteum following PGF$_{2\alpha}$ Administration**

Next, we addressed the question whether the ICAD sensitive DNase activity with a potential to cleave DNA into oligonucleosomes under *in vitro* conditions was indeed induced during PGF$_{2\alpha}$-induced luteal tissue apoptosis. As shown in Fig. 10A, this ICAD sensitive DNase activity was induced in the corpus luteum by PGF$_{2\alpha}$ treatment, associated with apoptotic DNA fragmentation. To further confirm the identity of the DNase as CAD, we carried out immunodepletion studies with the CAD antibody and results are presented in Fig. 10B. As can be seen in Fig. 10B, depletion of CAD from the corpus luteum tissue lysate collected 18h post PGF$_{2\alpha}$ treatment abolished the DNase activity in the supernatant (S). When the CAD immune complex pellet (P) was used in the DNase assay, it induced DNA fragmentation and the activity in the pellet was abrogated by addition of 100 ng of GST-ICAD-L. While IgG control did not show any activity in the immune complex, but the activity was still present in the supernatant demonstrating the specificity of the immunodepletion experiment. These results confirm the identity of the apoptotic endonuclease, CAD in mediating the PGF$_{2\alpha}$-induced DNA fragmentation in the luteal tissue.

**DISCUSSION**

Prostaglandin F$_{2\alpha}$ actions on the corpus luteum can be divided into two distinct, but interrelated processes; functional and structural luteolysis. The functional luteolysis which is characterized by rapid reduction in serum/luteal tissue progesterone levels may be viewed as the commencement of initiation of a new reproductive cycle due to the withdrawal of negative feedback effect of progesterone on the gonadotropin secretion. Whereas structural luteolysis that occurs as a consequence of apoptotic cell death is required for resorption of luteal structure in the ovarian stroma (48). Prostaglandin F$_{2\alpha}$ affects progesterone production either by interfering with the cholesterol transport to the inner mitochondrial membrane (49) or by enhancing catabolism of progesterone (50). In the rat, PGF$_{2\alpha}$ has been reported to enhance catabolism of progesterone rather than inhibit the synthesis and this action is brought about by the stimulatory effects of PGF$_{2\alpha}$ on Nur77-mediated increased expression of 20α-hydroxy steroid dehydrogenase, a key enzyme involved in progesterone catabolism (50). However, in the bovine corpus luteum, PGF$_{2\alpha}$ does not seem to have a significant effect on the stimulation of 20α-hydroxy steroid dehydrogenase and it is likely that the PGF$_{2\alpha}$-mediated decreased transport of cholesterol to inner mitochondrial membrane is the key mechanism resulting in decreased steroidogenesis (2). It is interesting to note that mitochondria might be playing an
important role in PGF$_{2\alpha}$-activated signal transduction pathways responsible for decreased steroidogenesis as well as structural luteolysis. In the present study we investigated spatial and temporal changes in the apoptotic signaling cascades in the luteal tissue of the buffalo cow, a bovine species, in which PGF$_{2\alpha}$ is recognized as the physiological luteolysin. In addition, a pseudopregnant rat model was utilized to investigate the relative contribution of intrinsic (mitochondrial) versus extrinsic (death receptor mediated) apoptotic signaling cascades in PGF$_{2\alpha}$-mediated luteal tissue apoptosis.

Studies carried out in several species have clearly established that apoptosis plays an important role during structural luteal regression (6-10). In order to elucidate the molecular events associated with the onset of apoptosis, it is necessary to select suitable time points with clear earliest evidence for onset of apoptosis. For this purpose, we employed two methods to determine initiation of apoptosis. The DNA fragmentation analysis reported earlier by us (7) and the in situ apoptosis analysis results reported in the present study indicated onset of apoptosis in the luteal tissue at 18h post PGF$_{2\alpha}$ treatment. In order to characterize changes leading to initiation of apoptosis, we included two additional time points (4 and 12h) earlier to 18h in which no evidence for apoptosis was evident, but at these time points the effect of PGF$_{2\alpha}$ on steroidogenesis was clearly discernible. These results broadly corroborate with the observation made by others with regard to the initiation of apoptosis in cattle and other species (6-10).

Great strides have been made in the recent years towards a better understanding of the intracellular signaling cascades that regulate the initiation of apoptosis in the cells exposed to apoptotic stimuli. One of the major intracellular signaling pathways that regulate apoptosis during cellular stress is mediated by changes in the Bcl-2 family of proteins that are classified either as anti-apoptotic (Bcl-2, Bcl-X$_\text{L}$ etc.,) or pro-apoptotic (Bax, Bad, etc.,) and it has been proposed that the fate of a cell at any given time is decided by the ratio or balance between the pro- and anti-apoptotic members (11). A luteolytic dose of PGF$_{2\alpha}$ brought about a rapid increase in the Bax levels (mRNA and protein), but without having much effect on the Bcl-2 levels (mRNA and protein) resulting in an increase in the Bax/Bcl-2 ratio. Higher Bax mRNA levels have been observed in the spontaneously regressing bovine corpus luteum by Rueda et al., (24) and results of the present study also confirms the importance of Bax in the luteal regression process and provides evidence for the regulation of Bax expression by PGF$_{2\alpha}$ in the corpus luteum. Apart from Bcl-2 and Bax, another Bcl-2 family member; Bad, plays an important role during apoptosis, and a decrease in the phosphorylation status of Bad has been shown to be associated with the apoptosis in the cells (51). Analysis of luteal tissue revealed hypophosphorylation of Bad as early as 4h that remained lower thereafter, suggesting its increased pro-apoptotic activity.

We next examined changes in the caspase-9 and 3, the activation of which has been linked to the initiation (caspase-9) and execution (caspase-3) of apoptosis, downstream to mitochondria (52). While the involvement of caspase-9 during luteal tissue apoptosis has not been reported in corpus luteum of any species thus far, several lines of evidence suggest that it plays an important role in the cellular apoptosis via cleavage mediated activation of its downstream target, caspase-3 (53). Caspase-3 in turn cleaves several downstream targets involved in cellular homeostasis viz., PARP, ICAD etc. In the present study, although total form of PARP increased in expression at 18h, cleaved form of PARP was only found to be upregulated at 18h following PGF$_{2\alpha}$ injection compared to levels in the corpus luteum of control animals, further confirming activation of caspase-3 in the process. In the murine corpus luteum Wang et al (54) have reported increased expression of PARP during PGF$_{2\alpha}$-induced luteal tissue apoptosis and this could be due to the increased stability of PARP mRNA or higher protein expression during luteolysis. Involvement of caspase-3 in mediating apoptosis in the mammalian cells is well established (52). Rueda et al., (55) found an increase in the caspase-3 activation in the ovine luteal tissue during PGF$_{2\alpha}$-induced luteolysis. Carambula et al., (23) reported that corpus luteum of caspase-3 deficient mice is resistant to PGF$_{2\alpha}$-mediated apoptosis that further confirms the involvement of caspase-3 in luteal cell apoptosis. Surprisingly, although the increase in the caspase-9 and -3 activation observed at 4-12h time points correlated with the expression changes in the Bcl-2 family members (increased Bax levels and decreased phospho-Bad levels), the dramatic induction in their activities at 18h, in
particular of caspase-3, could not be explained by changes in the expression or localization of the mitochondrial Bcl-2 family members, suggesting involvement of other pathways in the activation of caspase-3, in addition to caspase-9.

Apart from the intrinsic apoptotic signaling cascade, extrinsic (or death receptor mediated) apoptotic signaling cascade has been reported to play an important role in the regulation of apoptosis in various cell types exposed to a variety of apoptotic stimuli (12). The finding that Fas expression was induced at different time points post PGF$_{2\alpha}$ treatment suggested a role for Fas in luteal tissue apoptosis. Taniguchi et al., (25) also observed increased expression of Fas in the corpus luteum during the late luteal phase, a period in which spontaneous luteolysis would have set in. However, it has been reported that expression of Fas alone is not sufficient for inducing cell death and that FasL expression is the key determinant in regulating the activity of Fas in mediating apoptosis (56). The observation that FasL expression was induced in the luteal tissue at 18h post PGF$_{2\alpha}$ treatment suggests involvement of Fas-FasL in apoptosis of the luteal tissue, further confirmed by the observation of caspase-8 activation, which acts as an initiator caspase downstream to the Fas receptor. In a recent study, Carambula et al., (23) using induced multiple follicular growth and ovulation model reported that PGF$_{2\alpha}$ can induce activation of caspase-8 associated with the apoptosis in the murine corpus luteum, however, the upstream death receptor coupled to the activation of caspase-8 was not reported in their study. Our results in rat and bovine corpus luteum provide support for the importance of caspase-8 signaling in the luteal tissue apoptosis and strongly suggest that this is more likely due to the induction of FasL and Fas expression by PGF$_{2\alpha}$. Interestingly, activation of extrinsic apoptotic signaling cascade was only observed at 18h and this might provide an explanation for the induction in the caspase-3 activity at 18h time point since caspase-3 is a downstream target of caspase-8. However, it does not explain the amplification in the mitochondrial cascade observed at 18h. In this regard it has been proposed that activated caspase-8 cleaves Bid and this (tBid) can amplify the mitochondrial cascade by modulating the ratio of pro- to anti-apoptotic members at the level of mitochondria (57). Indeed, examination of the corpus luteum for tBid levels at different time points post PGF$_{2\alpha}$ treatment revealed an induction in the tBid levels only at 18h providing possible explanation for amplification in the caspase-9 activity observed at the 18h time point.

As it was impracticable due to the very large body size (> 400 kg), difficulty in accessing ovaries in vivo and quantity of inhibitors required to mechanistically address the involvement of intrinsic and extrinsic apoptotic signaling cascades in the PGF$_{2\alpha}$-induced luteal tissue apoptosis in the bovine model we investigated the relative contribution of these cascades in PGF$_{2\alpha}$-induced luteal tissue apoptosis using a well established pseudopregnant rat model (39, 58). Although PGF$_{2\alpha}$ has been reported to induce luteolysis in this model (39), the involvement of apoptotic signaling cascades in this model remains poorly characterized. Therefore, we first examined the involvement of intrinsic and extrinsic apoptotic signaling cascades during PGF$_{2\alpha}$-induced luteal tissue apoptosis in the pseudopregnant rats. Our results of increased expression of Bax and FasL, and activation of Caspase-9, 8 and 3, demonstrate for the first time PGF$_{2\alpha}$-induced activation of apoptotic signaling cascades in the rat corpus luteum, and that this model can be used to study the effect of pharmacological inhibition of activities of different caspases on PGF$_{2\alpha}$-induced luteal tissue apoptosis. Two peptide based cell permeable, irreversible caspase inhibitors were used to inhibit initiator caspase in intrinsic [caspase-9 (Z-LEHD-FMK)] or extrinsic [caspase-8 (Z-IETD-FMK)] apoptotic signaling cascades. In addition, a broad spectrum caspase inhibitor (Z-VAD-FMK) was used to demonstrate the effect of executionary caspase (caspase-1, 3, 4 and 7) inhibition on luteal tissue apoptosis. These peptide based caspase inhibitors have been demonstrated to inhibit caspase-dependent cell death in various in vivo models of apoptosis viz., cerebral ischemia (34, 35), myocardial dysfunction in sepsis (36), liver injury (37) and thymic dysfunction in sepsis (38). In the present study, the broad spectrum caspase inhibitor inhibited both caspase-3 activation and DNA fragmentation following PGF$_{2\alpha}$ injection in the luteal tissue. Caspase-8 inhibitor also could reduce PGF$_{2\alpha}$-induced caspase-3 activity and DNA fragmentation in the luteal tissue and the levels were similar to that observed in the luteal tissue of vehicle treated control animals, but caspase-9 inhibitor only had a marginal effect on these parameters. These results
suggest that extrinsic apoptotic signaling cascade plays an indispensable role during PGF$_{2\alpha}$-induced luteal tissue apoptosis. To our knowledge, this is the first demonstration of activation of classical apoptotic signaling cascades by an atypical death receptor via induction of upstream genes that activate these apoptotic signaling cascades under in vivo conditions. However, the involvement of other death receptors in this process can not be ruled out, as caspase-8 is also known to act downstream of many other receptors that induce cell death such as TNFα receptor (56).

DNA fragmentation is an important event in the executionary phase of cellular apoptosis (59). Although, several endonuclease like properties have been reported to be present in the luteal cells (29, 30), the endonuclease responsible for apoptotic DNA fragmentation in response to PGF$_{2\alpha}$ remains to be identified. We demonstrate presence of a Ca$^{++}$/Mg$^{++}$ dependent, temperature sensitive DNase maximally active at neutral pH conditions in the crude cytosolic extract prepared from luteal tissue collected at 18h post PGF$_{2\alpha}$ treatment. Moreover, we show that this putative DNase was induced by PGF$_{2\alpha}$ and was associated with the DNA fragmentation in the corpus luteum at 18h time point. The optimal DNase activity in the luteal cytosolic extract was observed at pH 6.5-7.5 ruling out the possible involvement of alkaline or acidic endonucleases (DNase I/II) or endonucleases that show two pH optima viz., Endonuclease-G during PGF$_{2\alpha}$-induced apoptotic DNA degradation in the corpus luteum. The biochemical nature of the DNase observed in the regressing corpus luteum was very similar to the properties of CAD reported by Nagata and coworkers (43). Caspase activated DNase has been reported to be activated during apoptosis by caspase-3 dependent cleavage of its inhibitor (ICAD). Examination of luteal tissue lysates for ICAD by western blotting revealed increased presence of a ~30 kDa ICAD reactive product at 12h that apparently reflects a partially processed intermediate observed by others (43, 47). The partially cleaved ICAD further decreased at 18h suggesting that degradation of ICAD at 18h post PGF$_{2\alpha}$ might be responsible for the activation of the caspase activated DNase in the luteal tissue resulting in the fragmentation of chromosomal DNA into 180 bp ladder. We reasoned that if this were to be true, addition of exogenously expressed ICAD should be able to abolish the DNase activity observed in the cytosolic extract prepared from PGF$_{2\alpha}$ treated luteal tissue. Indeed addition of GST-ICAD-L to the DNase assay abolished the DNase activity present in the cytosolic extract. In addition, immunodepletion of CAD from regressing luteal lysates abolished the DNA fragmentation activity observed in the regressing luteal lysates and the DNase activity observed in the immune complex was abolished by the addition of GST-ICAD-L in the DNase assay. Together, these data provide convincing evidence for the involvement of caspase activated DNase (CAD) in apoptosis associated with PGF$_{2\alpha}$-induced luteal tissue apoptosis.

Based on the findings in the present study and studies by others, we propose a model for the PGF$_{2\alpha}$-induced apoptotic signaling pathways in the corpus luteum (Fig. 10). Prostaglandin F$_{2\alpha}$-induced apoptotic signaling in the corpus luteum involves activation of stress activated protein kinases such as JNK and p38 MAP kinases (7, 60) that have been shown to result in the increased expression of genes that are primary initiators of apoptotic signaling machinery viz., Bax, FasL/Fas (61). Ligation of the FasL to Fas receptor or changes in the mitochondrial permeability results in the activation of the initiator (caspase-8 and -9) and executionary (caspase-3) caspases. Importance of caspase signaling in mediating luteal tissue apoptosis is further demonstrated in the present study by the use of caspase inhibitors, and in a previous study by the absence of luteal apoptosis in response to PGF$_{2\alpha}$ in caspase-3 knockout mice (23). These caspases then cleave key cellular proteins that include ICAD, resulting in the release of CAD, followed by the translocation of CAD to the nucleus that executes the DNA fragmentation. These apoptotic luteal cells are then recognized and cleared from the ovarian stroma by immune cells that have been shown to infiltrate in the ovary during luteolysis. In conclusion, the results from the present study suggest activation of both intrinsic and extrinsic apoptotic signaling cascades in the corpus luteum in response to PGF$_{2\alpha}$ treatment and there appears to be distinct phases in which each cascade is initiated, but eventually both converge to induce apoptosis.
REFERENCES


ACKNOWLEDGEMENTS

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and Mr. K. R. Shiva kumar for help with experiments in rats, and Mr. T. N. Vivek for help with expression and purification of the recombinant GST fusion proteins.

FIGURE LEGENDS

Figure 1. Prostaglandin F$_{2\alpha}$-induced apoptosis in the bovine corpus luteum.
(A), Schematic representation of the experimental procedure for PGF$_{2\alpha}$ treatment and corpus luteum collection in buffalo cows (*Bubalus bubalis*). * indicates time of collection of corpus luteum.
(B), Corpora lutea collected from the buffalo cows at different time points after PGF$_{2\alpha}$ treatment were processed for *in situ* apoptosis analysis using *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany). Arrows indicate apoptotic nuclei. Magnification 100x.

Figure 2. Prostaglandin F$_{2\alpha}$-induced changes in Bcl-2 and Bax mRNA and protein levels in the bovine corpus luteum.
(A), Total RNA isolated from the corpora lutea collected before and at different time points after PGF$_{2\alpha}$ treatment was subjected to semiquantitative RT-PCR analysis for Bcl-2 and Bax mRNA levels. L-19 mRNA expression was utilized as an internal control. The estimated sizes of PCR products (bp) are indicated on the left.
(B), Equal amounts of total protein lysates (200 µg/lane) were resolved on a 12% SDS-PAGE and transferred on to PVDF membrane for immunoblotting with Bax and Bcl-2 antibodies. As a control for protein loading the blot was stripped of the bound antibodies and reprobed with β-Actin antibody. The estimated sizes (kDa) of the protein bands are indicated on the left.
(C), Quantitative changes in the ratio of Bcl-2 and Bax mRNA (A) and Bcl-2 and Bax protein levels (B) in corpora lutea collected before and at different time points after PGF$_{2\alpha}$ treatment. Mean ± SEM of three experiments is shown. Bars with different letters are significantly (p < 0.05) different.
(D), Representative corpora lutea sections of immunohistochemical staining for Bax. Corpora lutea sections from the luteal tissue collected before and at different time points after PGF$_{2\alpha}$ treatment were rehydrated and incubated with Bax antibody followed by incubation with FITC conjugated anti-rabbit antibody raised in goat. Sections were mounted in glycerol and visualized in confocal microscope at 100x (Z 1.5). Inset shows immunostaining observed with rabbit IgG (negative control).

Figure 3. PGF$_{2\alpha}$-induced regulation of processed caspase-9 protein levels and activity in the bovine corpus luteum.
(A), Equal amounts of total protein lysate (200 µg/lane) prepared from corpora lutea collected before and at different time points after PGF$_{2\alpha}$ treatment were resolved on a 12% SDS-PAGE and transferred on to PVDF membrane for immunoblotting. Membrane was probed with antibody that detects endogenous levels of large fragment (37 kDa) of cleaved caspase-9 and the blot was stripped of the bound cleaved caspase-9 antibody and reprobed with β-Actin antibody to confirm equal loading of protein across lanes. Mean ± SEM of cleaved caspase-9 protein levels relative to β-Actin are shown below the western blot image.
(B), Caspase-9 activity assay on luteal tissue lysates using Z-LEHD-AFC as a substrate indicating changes in caspase-9 activity in corpora lutea collected before and at different time points after PGF$_{2\alpha}$ treatment. Specificity of the assay was checked using caspase-9 specific inhibitor Z-LEHD-FMK (see materials and methods for details of the assay). Mean ± SEM of three experiments is shown. Bars with different letters are significantly (p < 0.05) different.
Figure 4. Prostaglandin F$_2\alpha$-induced regulation of processed caspase-3 protein levels and activity in the bovine corpus luteum.

(A), Equal amounts of total protein lysate (200 µg/lane) prepared from corpora lutea collected before and at different time points after PGF$_2\alpha$ treatment were resolved on a 12% SDS-PAGE and transferred on to PVDF membrane for immunoblotting. Membrane was probed with antibody that detects endogenous levels of large fragment (19/17 kDa) of cleaved caspase-3. The blot was stripped of the bound caspase-3 antibody and reprobed with β-Actin antibody to confirm equal loading of protein across lanes. Cleaved caspase-3 protein levels relative to β-Actin are indicated below the western blot image. Changes in the PARP cleavage as determined by western blotting, before and at different time after PGF$_2\alpha$ treatment is shown.

(B), Caspase-3 activity assay on luteal tissue lysates using Z-DEVD-AFC as a substrate indicating changes in caspase-3 activity in corpora lutea collected before and at different time points after PGF$_2\alpha$ treatment. Specificity of the assay was checked using caspase-3 specific inhibitor Ac-DEVD-CHO (see materials and methods for details of the assay). Mean ± SEM of three experiments is shown. Bars with different letters are significantly (p < 0.05) different.

Figure 5. PGF$_2\alpha$-induced changes in the extrinsic apoptotic signaling cascade elements in the bovine corpus luteum.

(A), Total RNA isolated from the corpora lutea collected before and at different time points after PGF$_2\alpha$ treatment was subjected to semiquantitative RT-PCR analysis for FasL and Fas mRNA levels. As a control for RNA amount variation in samples, same cDNA preparation was analysed for L-19 mRNA levels. The estimated sizes of PCR products (bp) are indicated on the left.

(B), Western blot analysis using Bid antibody shows tBid levels in the corpora lutea collected before and at different time points after PGF$_2\alpha$ treatment. Blot was stripped of the bound Bid antibody and reprobed with β-Actin antibody for confirmation of equal loading of protein across lanes.

(C), Caspase-8 activity assay on luteal tissue lysates using Z-IETD-AFC as a substrate indicating changes in caspase-8 activity in corpora lutea collected before and at different time points after PGF$_2\alpha$ treatment. Specificity of the assay was checked using caspase-8 specific inhibitor Z-IETD-FMK (see materials and methods for details of the assay). Mean ± SEM of three experiments is shown. Bars with different letters are significantly (p < 0.05) different.

Figure 6. Characterization of apoptotic signaling events during PGF$_2\alpha$-induced luteal tissue apoptosis in the pseudopregnant rats.

(A), Schematic representation of the experimental procedure for induction of luteal tissue apoptosis in the pseudopregnant rat model. * indicates time of collection of corpora lutea.

(B), Genomic DNA (30µg) isolated from corpora lutea, obtained 24h after vehicle or PGF$_2\alpha$ analog injection on day 8 of pseudopregnancy, was separated on 2% agarose gel and stained with ethidium bromide. Note the presence of DNA oligonucleosomes in the DNA isolated from PGF$_2\alpha$ treated animals.

(C), Semiquantitative RT-PCR analysis of Bax and FasL mRNA levels for vehicle/PGF$_2\alpha$ treated luteal tissue. L-19 mRNA expression was utilized as an internal control. Bar diagram represents quantitative changes in the mRNA levels relative to L-19.

(D-F), Caspase-9,-8 and -3 activity assays on luteal tissue lysates using substrates specific to respective caspases. For details, see legend to Figures 3-5.

Figure 7. Effects of caspase inhibitors on PGF$_2\alpha$-induced changes in the apoptotic signaling in the corpus luteum.

(A), Schematic representation of the experimental procedure to investigate the effect of caspase
inhibitors on PGF2α-induced luteal tissue apoptosis in the pseudopregnant rat model. * indicates
time of collection of corpora lutea.

(B), Determination of caspase-3 activity. DEVDase enzyme activity corresponding to caspase-3
activity was measured in the luteal tissue, 24h after in vivo treatments. Fifteen minutes prior to
s.c., injection of vehicle α PGF2α treatment, rats received intrabursal injection of vehicle or
specific caspase inhibitors (Z-LEHD-FMK, an caspase-9 inhibitor; or Z-IEPD-FMK, an caspase-
8 inhibitor; or Z-VAD-FMK, an general caspase inhibitor) as described in materials and methods.
Results are presented as arbitrary fluorescence units (AFU). Mean ± SEM of three experiments is
shown. Bars with different letters are significantly (p < 0.05) different. Different treatments are
indicated at the bottom of panel D.

(C), Qualitative changes in DNA fragmentation. DNA (30µg) isolated from luteal tissue, before
and 24h after different treatments (For details, see legend to Fig. 7B) was subjected to agarose gel
electrophoresis followed by ethidium bromide staining. Different treatments are indicated at the
bottom of the gel.

(D), Quantitative changes in DNA fragmentation. Low molecular weight DNA fragments (Panel
C) were quantified by densitometric analysis. Results (fold change from vehicle treatment) are
presented as arbitrary densitometric units. Mean ± SEM of three experiments is shown. Bars with
different letters are significantly (p < 0.05) different. Different treatments are indicated at the
bottom of the bars.

Figure 8. DNA fragmentation induced by PGF2α-activated luteal cytosolic extract.

(A), pH dependence of DNA fragmentation. Equal amount of cytosolic extract protein (50 µg)
prepared from the corpus luteum collected at 18h after PGF2α treatment was added to the nuclei
(3 X 10^6) from normal rat liver and incubated at 37°C for 30 min in the DNase assay buffer at
indicated pH values. DNA was extracted and analyzed by agarose gel electrophoresis. The
position of standard DNA fragments (bp) are indicated on the left.

(B) Dose-dependent DNase fragmentation. The cytosolic extract prepared from the corpus luteum
collected at 18h after PGF2α treatment at the indicated protein amounts was added to the isolated
nuclei (3 X 10^6) from normal rat liver followed by incubation at 37°C for 30 min in the DNase
assay buffer at pH 7.5. DNA was extracted and analyzed by agarose gel electrophoresis.

(C), Time course of DNA fragmentation. Cytosolic extract protein (50 µg) prepared from the
corpus luteum collected at 18h after PGF2α treatment was added to the isolated nuclei (3 X 10^6)
from normal rat liver and incubated at 37°C for indicated time periods in DNase assay buffer at
pH 7.5. DNA was extracted and analyzed by agarose gel electrophoresis. The standard DNA
fragments (lane M) are indicated on the left as base pairs (bp).

(D), Ca++/Mg++ dependent protein in the luteal cytosolic extract is responsible for inducing DNA
fragmentation. Cytosolic extract protein (50 µg) prepared from the corpus luteum collected at 18h
after PGF2α treatment was preincubated at 37°C for 60 min with 100 µg/ml RNase A or 68°C for
10 min (heat) or 5mM EGTA + 5mM EDTA at 37°C for 10 min Isolated nuclei (3 X 10^6) from
normal rat liver were added to the treated cytosolic extracts and incubated at 37°C for 30 min in
DNase assay buffer (pH 7.5). DNA was extracted and analyzed by agarose gel electrophoresis.

As a positive control for DNase activity, cytosolic extract (50 µg) from luteal tissue was
incubated with the isolated nuclei (3 X 10^6) without any preincubation (lane 1).

Figure 9. Inhibition of DNase activity by recombinant Inhibitor of Caspase Activated DNase
(ICAD).

Equal amount (50 µg) of cytosolic protein extract prepared from the corpus luteum collected 18h
after PGF2α treatment were preincubated with indicated amounts of GST-ICAD-L (Lanes 4-13)
and the DNase activity was determined with isolated nuclei (3 X 10^6) from normal rat liver (Top
panel) or 10 µg of plasmid DNA (Bottom panel). The effect of control GST protein on the DNase
activity present in the cytosolic protein extract is also shown (lane 14). Lane 2 shows integrity of
the nuclei/plasmid DNA used for the assay and lane 3 shows DNA fragmentation activity of the cytosolic extract without preincubation with GST/GST-ICAD-L. The position of standard DNA fragments (bp) are indicated on the left.

**Figure 10. Changes in DNA fragmentation activity in luteal tissue during PGF$_{2\alpha}$-induced apoptosis.**

(A), Equal amount of cytosolic protein extract (50 $\mu$g) prepared from the corpus luteum collected before and at 18h after PGF$_{2\alpha}$ treatment was added to the 10 $\mu$g of plasmid DNA (used as naked DNA substrate; left panel) or to the isolated nuclei (3 X 10$^6$) from normal rat liver (used as a chromatin substrate; right panel) and incubated at 37°C for 30 min in DNase assay buffer (pH 7.5). DNA was extracted and analyzed by agarose gel electrophoresis. The standard DNA fragments (bp, lane M) are indicated on the left.

(B), Immunodepletion of CAD from luteal tissue lysates. CAD was immunoprecipitated from 400$\mu$g of luteal tissue lysates and the resultant supernatant (S) and the CAD immune complex pellet (P) was used in the DNase assay as described in materials and methods. IgG supernatant and pellet was used as a control for the assay. Please note the presence of DNA fragmentation activity in the CAD pellet and abolition of DNA fragmentation activity in the CAD pellet with addition of 100ng of GST-ICAD-L.

**Figure 11.** A schematic representation of PGF$_{2\alpha}$-activated apoptotic signaling cascades in the bovine corpus luteum. Dashed lines (-----) represent results reported by others and solid lines (------) represent results reported in the present study. The detailed explanation is provided in the discussion section.
Figure 1

A

Day 11 Post-estrus

PGF$_{2\alpha}$ injection

Time (h) After PGF$_{2\alpha}$ injection

0* 4* 12* 18*

B

0h 4h 12h 18h

The images show changes over time after PGF$_{2\alpha}$ injection.
Figure 2

A

Time (h) After PGF$_{2\alpha}$ injection

<table>
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</tr>
<tr>
<td>237</td>
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<td></td>
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</tr>
<tr>
<td>128</td>
<td>Bcl-2</td>
<td></td>
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<tr>
<td>406</td>
<td>L-19</td>
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</table>

B

~kDa

| 21  | Bax  |     |     |     |
| 29  | Bcl-2 |     |     |     |
| 42  | β-Actin |     |     |     |

C

Bax/ Bcl-2 ratio (mRNA/Protein)

- mRNA
- Protein

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D

0h   4h   12h   18h

0h

4h

12h

18h
Figure 3

A

Cleaved Caspase-9

β-Actin

~kDa

0 4 12 18 Time (h)

B

Caspase-9 Activity (Fold increase vs 0h)

- Z-LEHD-FMK

+Z-LEHD-FMK

Time (h) After PGF$_{2α}$ injection

Relative Levels

0.14±0.1 0.64±0.2 0.58±0.1 1.41±0.3

a

b

b

c

0 1 2 3 4

0 4 12 18
Figure 4

A

- kDa

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>4</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>cleaved Caspase-3</td>
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<tr>
<td>0.51±0.2</td>
<td>0.74±0.1</td>
<td>0.57±0.1</td>
<td>1.64±0.3</td>
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B

- Actin

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<th>12</th>
<th>18</th>
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<tr>
<td>cleaved PARP</td>
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<tr>
<td>0.51±0.2</td>
<td>0.74±0.1</td>
<td>0.57±0.1</td>
<td>1.64±0.3</td>
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Caspase-3 Activity (Fold increase vs 0h)

<table>
<thead>
<tr>
<th>Time (h) After PGF$_{2x}$ injection</th>
<th>0</th>
<th>4</th>
<th>12</th>
<th>18</th>
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<tbody>
<tr>
<td>- Ac-DEVD CHO</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ac-DEVD CHO</td>
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Figure 5

A

<table>
<thead>
<tr>
<th>bp</th>
<th>0</th>
<th>4</th>
<th>12</th>
<th>18</th>
<th>Time (h)</th>
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<td>Fas</td>
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<td></td>
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<td>L-19</td>
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B

<table>
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<tr>
<th>~kDa</th>
<th>15</th>
<th>42</th>
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<td>tBid</td>
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<tr>
<td>β-Actin</td>
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</tbody>
</table>

C

Caspase-8 Activity (Fold increase vs 0h)

- Z-IETD-FMK
+Z-IETD-FMK

Time (h) After PGF$_{2α}$ injection

- a
- b
Figure 6

A
Vehicle/ PGF$_{2\alpha}$ inj.

Day 8

24h

Day 9*

B

Vehicle
PGF$_{2\alpha}$

C

bp
237
174
406

Bax
FasL
L-19

Relative Levels

Vehicle
PGF$_{2\alpha}$

D

Caspase-9 Activity

- Z-LEHD-FMK
+Z-LEHD-FMK

E

Caspase-8 Activity

- Z-IETD-FMK
+Z-IETD-FMK

F

Caspase-3 Activity

- Ac-DEVD-CHO
+Ac-DEVD-CHO
Figure 7

A

Vehicle/Cas. Inhibitor inj.  Vehicle/PGF$_{2\alpha}$ inj.  24h
Day 8 1030h 1045h Day 9*

B

Caspase-3 Activity (AFU)

D

DNA Fragmentation (Fold Increase)

<table>
<thead>
<tr>
<th>PGF$_{2\alpha}$</th>
<th>Z-LEHD-FMK</th>
<th>Z-IETD-FMK</th>
<th>Z-VAD-FMK</th>
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</thead>
<tbody>
<tr>
<td>-</td>
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<tr>
<td>+</td>
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</tbody>
</table>

Legend:
- DEVD-CHO
- +DEVD-CHO

C

DNA Fragmentation Gel Image
Figure 8

A

bp

3000
2000
1000
900
500
200

0
5
10
20
30
60

M

pH 4
pH 5
pH 6
pH 6.5
pH 7.5
pH 8.0
pH 9

Time (minutes)

B

Protein (μg)

0
25
50

C

bp

5148
2027
564

M

0
5
10
20
30
60
M

D

Luteal Lysate
Heat
EDTA/EGTA
RNase A
Figure 9

GST: 200 ng

GST-ICAD-L (ng)

DNA Integrity

bp

M  DNA Integrity  200  100  50  30  20  10  6  2  0.6  0.2  GST: 200 ng