Prostaglandin F$_{2\alpha}$-mediated Activation of Apoptotic Signaling Cascades in the Corpus Luteum during Apoptosis

INVIOLEMENT OF CASPASE-ACTIVATED DNase

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Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) 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$_{2\alpha}$-induced luteolysis in the bovine species in which initiation of apoptosis was demonstrable at 18 h after exogenous PGF$_{2\alpha}$ treatment. An analysis of intrinsic arm of apoptotic signaling cascade elements revealed that PGF$_{2\alpha}$ injection triggered increased ratio of Bax to Bcl-2 in the luteal tissue as early as 4 h posttreatment that remained elevated until 18 h. This increase was associated with the elevation in the active caspase-9 and -3 protein levels and activity ($p<0.05$) at 4–12 h, but a spurt in the activity was seen only at 18 h posttreatment 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 18 h accompanied by a significant ($p<0.05$) induction in the caspase-8 activity and truncated Bcl levels. Furthermore, 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$_{2\alpha}$ treatment. The DNase responsible for PGF$_{2\alpha}$-induced apoptotic DNA fragmentation was found to be Ca$_{\text{II}}$/Mg$_2$-dependent, temperature-sensitive DNase, and optimally active at neutral pH conditions. This putative DNase was inhibited by the recombinant inhibitor of caspase-activated DNase, and immunodepletion of caspase-activated DNase 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$_{2\alpha}$-induced apoptosis in the corpus luteum.

Although PGF$_{2\alpha}$ was discovered as a physiological luteolysin nearly three decades ago (1), cellular events associated with luteolysis remain poorly characterized, in part because of the lack of availability of a suitable in vitro model system that mimics all of the cellular events that occur in vivo in response to spontaneous or PGF$_{2\alpha}$-induced luteolysis (2). Prostaglandin F$_{2\alpha}$ interacts with its G protein-coupled receptor, present predominantly on large luteal cells, but are also present on small luteal and endothelial cells of the corpus luteum (3) and activates G$_{\text{q}}$/phospholipase C/protein kinase C pathway (4, 5), resulting in decreased steroidogenesis. The 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$_{2\alpha}$-induced or spontaneous luteolysis of several species (6–10).

Apoptosis or programmed cell death is an evolutionarily conserved mechanism orchestrated by the genome-encoded proteins of the host 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-apoptotic 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/Inhibitor of caspase-activated DNase (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 a 180-bp DNA ladder, which is considered as one of the hallmarks of cellular apoptosis. Candidates for such endonucleases

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include the caspase-activated enzymes, such as DFF40/ caspase-activated DNase (CAD) (14, 15) and Nuc70 (16), divalent cation-dependent neutral (17) or acidic endonucleases (18), and Ca²⁺/Mg²⁺-dependent endonucleases (19–21). Recently, however, another DNase, endonuclease 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, PGF₂α receptor is a G protein-coupled receptor that lacks any intracytoplasmic region with an identity/similarity to the death domain/caspase activation and recruitment domain that recruits and/or activates caspases. However, caspase-8, one of the initiator caspases acting downstream of the Fas receptor pathway, has recently been reported to be activated during PGF₂α-induced luteal tissue apoptosis in the corpus luteum of murine species (23) and the authors hypothesized that some level of cross-talk exists between PGF₂α 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, whereas 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), interferon-γ (27), and tumor necrosis factor-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 PGF₂α-induced apoptosis in the luteal tissue has not been identified, although several DNase-like activities, viz. Ca²⁺/Mg²⁺-dependent DNase (29), Zn²⁺-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 PGF₂α remains to be determined.

The purpose of this study was to systematically analyze the involvement of classical apoptotic signaling cascades during PGF₂α-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 PGF₂α 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), because the characterization of apoptosis following spontaneous or PGF₂α-induced luteolysis in this species was reported recently (7). Our results show for the first time that PGF₂α-mediated apoptosis in the corpus luteum involves the activation of mitochondrial apoptotic signaling cascade that precedes the activation of FasL/Fas cascade during PGF₂α-induced apoptotic cell death in the bovine corpus luteum. A pseudopregnan rat model was standardized to investigate the relative roles of intrinsic and extrinsic apoptotic signaling cascades in PGF₂α-induced luteal tissue apoptosis. Using the pseudopregnan rat model, we demonstrate that intrabursal injection of a peptide-based cell-permeable caspase-8 inhibitor (Z-IETD-fmk) prior to PGF₂α injection abolished the caspase-3 activity and DNA fragmentation during PGF₂α-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 CAD in mediating apoptotic DNA fragmentation during PGF₂α-induced luteolysis.

**EXPERIMENTAL PROCEDURES**

**Reagents**

The polyclonal antibodies specific to Bcl-2 (number 197207), Bax (number 198820), phospho-BAD (number 9291), BAD (number 9292), cleaved caspase-9 (number 9501S), cleaved caspase-3 (number 9661S), Bid (number 2002), and ICAD (number PC366T) were purchased from Calbiochem (numbers 197207, 198820, and PC366T) and Cell Signaling Technology (Beverly, MA) (numbers 9291, 9292, 9501S, 9661S, and 2002). PARP antibody (AM30) was purchased from Oncogene Research Products (Boston, MA). Caspase-activated DNase antibody (Ab5406) was purchased from Abcam Inc. (Cambridge, MA). β-Actin antibody (A5441) was purchased from Sigma. Caspase substrates and inhibitors (Z-DEVD-APC (caspase-3 substrate 264150), Z-IEHD-APC (caspase-8 substrate 368059), Z-LEHD-APC (caspase-9 substrate 218765), Ac-DEVD-CHO (caspase-3 inhibitor 235420), Z-LEOMe/TDO/OMe-fmk (caspase-8 inhibitor 218759), Z-LEOMe/HDX/OMe-fmk (caspase-9 inhibitor 218761), and Z-VAD/OMe-fmk (General executionary caspase (caspase-1, -3, -4, and -7) inhibitor 627610) were purchased from Calbiochem. All reagents were obtained from Amresco Biosciences. Membrane and PVD membranes were purchased from PerkinElmer Life Sciences. All of the other reagents were purchased from Sigma or Invitrogen or sourced locally.

**Animal Models and Methods**

A. **PGF₂α-induced Luteal Tissue Apoptosis in the Buffalo Cores (B. bubalis)—**All of the procedures in animals were approved by the Institutional Animal Ethics Committee (Indian Institute of Science). Serum withdrawal (27), because the characterization of apoptosis following spontaneous or PGF₂α-induced luteolysis in this species was reported recently (7). Our results show for the first time that PGF₂α-mediated apoptosis in the corpus luteum involves the activation of mitochondrial apoptotic signaling cascade that precedes the activation of FasL/Fas cascade during PGF₂α-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 PGF₂α-induced luteal tissue apoptosis. Using the pseudopregnan rat model, we demonstrate that intrabursal injection of a peptide-based cell-permeable caspase-8 inhibitor (Z-IETD-fmk) prior to PGF₂α injection abolished the caspase-3 activity and DNA fragmentation during PGF₂α-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 CAD in...
luted in 0.3 ml of 0.15 M NaCl or 0.3 ml of 0.15 M NaCl (vehicle) treatment. The animals were killed 24 h 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 this study were prepared based on the previous studies where as few 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 PGF₂α employed in this study was based on a previous study in the pseudopregnant rat model (39).

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

RNA Extraction and Semi-quantitative 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 were assessed spectrophotometrically and on a 1% formaldehyde-agarose gel. Semi-quantitative RT-PCR was carried out essentially as described previously (40). Oligonucleotide primers were designed for Bax, FasL, Fas, and L-1 genons based on the conserved regions present in mouse RNA sequences of Homo sapiens, Bos taurus, and Mus musculus. The primers (forward and reverse) and PCR conditions (annealing temperature and PCR cycle number) used were as follows: 5′-TACCTCAGGGATGACGAGGG-3′ and 5′-CGGCCCAGGTTAAAGTTGCC-3′ for 237-bp Bax (56 °C and 35 cycles); 5′-CTCTGAGT-GATCGTAATGACC-3′ and 5′-GAGACGAGCAGGAAGAAATC-3′ for 128-bp Bcl-2 (53 °C and 35 cycles); 5′-AGGCTGTCACCCCAAGTCAC-CC-3′ and 5′-GAGCCCAGTTTCTAGGTCACAAAGC-3′ for 174-bp Fasl (55 °C and 35 cycles); 5′-AGGGGAAAGCTGATCACAGAC-3′ and 5′-GCAAAGGGTTATGAGTTCACC-3′ for 178-bp FasL (50 °C and 35 cycles); and 5′-GAAATCTGCAAGTTCAATC-3′ and 5′-TTTGCACGTCGAGCTTCA-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 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 of protein/plane) was resolved by 10 or 12% SDS-PAGE and 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 software.

Caspase-8/9 Activity Assays
Caspase-8/9 activity assays were performed using fluorogenic substrates Z-IETD-AFC (caspase-8) and Z-LEHD-AFC (caspase-9). Luteal tissues collected before and after PGF₂α treatment and after coinjection (15 min apart) of caspase inhibitors and PGF₂α were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycine, and 1 mM NaVO₄). After determining the protein concentration, an equal amount (200 µg) of luteal tissue protein (at non-saturating concentration of the assay, for details see Supplemental Fig. 1) was loaded into 1 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 1 h, and liberated AFC was measured in a fluorometer with an excitation wavelength of 400 nm and an emission wavelength of 420–520 nm. The specificity of the caspase-3 assay was determined by using caspase-3-specific inhibitor Ac-DEVD-CHO.

Deoxyribonuclease Substrates
Caspase-8/9 Activity Assays
For DNase assays crude cytosolic extracts were prepared from the luteal tissue essentially as described previously (43, 44). Luteal tissue collected before and at 18 h post-PGF₂α treatment was swollen in an equal volume of extraction buffer (50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 20 µM μtcholisin B, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) for 10 cycles of freezing and thawing, which was accompanied by grinding with a pestle each time. The resulting lysate was then centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was aspirated, aliquoted, and frozen at −70 °C until use 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 minor 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 (with the exception of 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, and 0.1 mM phenylmethylsulfonyl fluoride) remaining the same. Assay was terminated by the addition of 6× 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 × 10⁶ 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 mM NaCl, 0.2% SDS, and 0.1 mg/ml protamine K), and DNA was precipitated after overnight incubation at 37 °C by the addition of an equal volume of isopropl alcohol. 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 re- (18 h after PGF₂α treatment) luteal luteal protein with polyclonal anti-CAD antibody or rabbit IgG and protein A-agarose at 4 °C overnight. The pellet containing immune complexes was obtained by centrifugation at 15,000 × 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 DNAase activity assay. However, supernatant 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
Wherever applicable, the data were expressed as mean ± S.E. The arbitrary densitometric units were represented as the percentage rela-
activity during PGF2α determined changes in the active caspase-9 protein levels and decrease in the phosphorylation of Bad within 4 h, and the phosphorylation, was also examined in the luteal tissue that showed a Bcl-2 family member that gets activated upon hypophosphorylation. However, IgG control did not show any staining (shown in Fig. 1B). Although no incidence of apoptosis was observed in the corpus luteum at 4 h, some evidence of apoptosis was present in the corpus luteum at 12 h after PGF2α injection. These data confirm our previous observation of the time of onset of apoptotic DNA fragmentation during PGF2α-induced luteolysis (7) and further validate 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 PGF2α-induced Apoptosis**

Fig. 2 illustrates changes in steady-state mRNA and protein levels of pro- and anti-apoptotic Bcl-2 family members during PGF2α-induced luteal tissue apoptosis. Bax (a pro-apoptotic gene) mRNA (Fig. 2A) and protein levels (Fig. 2B) increased within 4 h and remained high at 18 h, 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-PGF2α treatment (Fig. 2, A and B). Changes in Bax and Bcl-2 mRNA and protein levels were 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 4 h, which remained high until 18 h, 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 PGF2α-induced luteal tissue apoptosis, a parameter that confirms increased localization of proteins to mitochondria (45). However, IgG control did not 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 a decrease in the phosphorylation of Bad within 4 h, and the levels remained low at 18 h (Supplemental Fig. 2A). We next determined changes in the active caspase-9 protein levels and activity during PGF2α-induced luteal tissue apoptosis. As seen in Fig. 3A, active caspase-9 protein levels increased in the luteal tissue 4–12 h after PGF2α treatment and were highest at 18 h.

**Activation of Caspase-3 in the Corpus Luteum during PGF2α-induced Apoptosis**

To address whether caspase-9 activation (initiator caspase) was associated with an increase in the activation of downstream target effector caspase, caspase-3, we measured the 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 18 h. An 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 18 h post-PGF2α treatment (Fig. 4B). The 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**

Fig. 5 illustrates changes in steady-state mRNA levels of Fas and FasL in the corpus luteum before and at different time points after PGF2α treatment. As can be seen in Fig. 5A, FasL mRNA expression levels were unchanged in the corpus luteum at 4 and 12 h but the expression was high at 18 h. The Fas receptor mRNA levels were up-regulated in the corpus luteum within 4 h (4.5-fold compared with 0 h) and became low at 12 h (1.4-fold compared with 0 h) but increased again at 18 h (2.3-fold compared with 0 h). An analysis of caspase-8 activation revealed no change at 4–12 h posttreatment, but the activity increased significantly (p < 0.05) at 18 h (Fig. 5C). Z-LETD-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). We next determined changes in the 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. 5B. Whereas tBid was undetectable at 0, 4 and 12 h, the signal was high at 18 h post-PGF2α treatment.

**Relative Contribution of Intrinsic and Extrinsic Apoptotic Signaling Cascades in PGF2α-induced Apoptosis**

We next investigated the relative contribution of intrinsic and extrinsic apoptotic signaling cascades in mediating PGF2α-induced luteal tissue apoptosis. For this purpose, we standardized a pseudopregnant rat model for PGF2α-induced luteolysis and employed a pharmacological blockade of specific caspases associated with intrinsic and extrinsic apoptotic signaling cascades as well as general caspases using peptide-based, cell-permeable, and irreversible caspase inhibitors. Before analyzing the effects of caspase inhibition on PGF2α-induced luteal tissue apoptosis in pseudopregnant rats, it was necessary to determine whether PGF2α 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, semi-quantitative RT-PCR analysis of upstream activators of intrinsic and extrinsic apoptotic signaling cascades (Bax and FasL, respectively), and changes...
PGF<sub>2α</sub>-activated Apoptotic Signaling Cascades in Corpus Luteum

Fig. 2. Prostaglandin F<sub>2α</sub>-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<sub>2α</sub> treatment was subjected to semi-quantitative 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 (in 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 onto 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 (in 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<sub>2α</sub> treatment. Mean ± S.E. of three experiments is shown. Bars a–c are significantly different (p < 0.05). D, representative corpora lutea sections from the luteal tissue collected before and at different time points after PGF<sub>2α</sub> treatment were rehydrated and incubated with Bax antibody followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit antibody raised in goat. Sections were mounted in glycerol and visualized under a confocal microscope at ×100 (Zoom 1.5). Inset shows immunostaining observed with rabbit IgG (negative control).

Fig. 3. PGF<sub>2α</sub>-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<sub>2α</sub> treatment were resolved on a 12% SDS-PAGE and transferred 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 ± S.E. 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<sub>2α</sub> treatment. Specificity of the assay was checked using caspase-9-specific inhibitor Z-LEHD-fmk (see “Experimental Procedures” for details of the assay). Mean ± S.E. of three experiments is shown. Bars a–c are significantly different (p < 0.05).

in caspase-9, -8, and -3 activities during PGF<sub>2α</sub>-induced luteal tissue apoptosis are presented in Fig. 6, B–F. As can be seen in Fig. 6B, increased DNA oligonucleosome formation is discernible in the DNA isolated from luteal tissues collected 24 h after PGF<sub>2α</sub> injection, indicative of cell death through apoptosis. Semi-quantitative RT-PCR analysis revealed up-regulation in the steady-state mRNA levels of Bax and FasL during PGF<sub>2α</sub>-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 are associated with the activation of downstream caspases. The results of caspase activation analysis are presented in Fig. 6, D–F. As can be seen from the figures, a significant (p < 0.05) up-regulation in caspase-9 (1.9 ± 0.04-fold versus 0 h), caspase-8 (3.5 ± 0.5-fold versus 0 h), and caspase-3 (4.8 ± 0.3-fold versus 0 h) activities occurred in the luteal tissue following PGF<sub>2α</sub> 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<sub>2α</sub>-induced luteal tissue apoptosis in vivo.

Fig. 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<sub>2α</sub>-induced luteal tissue apoptosis, and changes in caspase-3 activities and oligonucleosome formation in the luteal tissue are presented in Fig. 7, B–D. Although caspase-9 inhibitor significantly (p < 0.05) decreased PGF<sub>2α</sub>-induced caspase-3 activity, DNA oligonucleosome formation was only marginally decreased in the luteal tissue. In contrast, the administration of caspase-8 inhibitor or general caspase inhibitor resulted in dramatic reduction in PGF<sub>2α</sub>-induced caspase-3 activity and oligonucleosome formation. These results suggest the requirement of activation of extrinsic apoptotic signaling cascade, which is necessary and sufficient for PGF<sub>2α</sub>-induced
apoptosis in the luteal tissue, whereas the activation of intrinsic apoptotic signaling cascade alone is insufficient.

**Induction of a Ca^{2+}/Mg^{2+}-dependent DNase Activity in Corpus Luteum during PGF_{2\alpha}-induced Apoptosis**

To examine the DNase activity responsible for apoptotic DNA ladder formation during PGF_{2\alpha}-induced luteal tissue apoptosis, we analyzed cellular DNase activity in the corpus luteum collected 18 h post-PGF_{2\alpha}. Before subjecting the PGF_{2\alpha}-treated tissues for characterization of DNase activity, tissues were analyzed for DNA oligonucleosome formation (Supplemental Fig. 3) to confirm the presence of DNase activity in corpus luteum collected from the buffalo cows 18 h post-PGF_{2\alpha} injection. Biochemical characterization of the DNase activity in the crude cytosolic extract of corpus luteum collected 18 h 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, 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^{2+}/Mg^{2+} 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 ICAD**

We next 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 partial ICAD fragments (46, 47) were observed following PGF_{2\alpha} treatment in the corpus luteum (Supplemental Fig. 4). The chemical properties of ICAD in association with the degradation of ICAD suggested that it could be CAD. To examine this possibility, ICAD-L, a specific inhibitor of CAD, was expressed in *Escherichia coli* as a GST fusion protein and purified as described previously by Nagata and co-workers (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 18 h post-PGF_{2\alpha} treatment in a dose-dependent fashion. Complete inhibition was observed at a dose of 100 ng, whereas 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$_{2a}$ Administration

We next addressed the question of whether the ICAD-sensitive DNase activity with a potential to cleave DNA into oligonucleosomes under in vitro conditions was indeed induced during PGF$_{2a}$-induced luteal tissue apoptosis. As shown in Fig. 10A, this ICAD-sensitive DNase activity was induced in the corpus luteum by PGF$_{2a}$ 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. These results are presented in Fig. 10B. As can be seen in Fig. 10B, depletion of CAD from the corpus luteum tissue lysate collected 18 h post-PGF$_{2a}$ treatment abrogated the DNA fragmentation activity in the lysate supernatant (Fig. 10, 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 the addition of 100 ng of GST-ICAD-L. Although IgG control did not show any activity in the immune complex, 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$_{2a}$-induced DNA fragmentation in the luteal tissue.

DISCUSSION

Prostaglandin F$_{2a}$ 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$_{2a}$ 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$_{2a}$ 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$_{2a}$ on Nur77-mediated increased expression of 20/H$_{2}$-hydroxy steroid dehydrogenase, a key enzyme involved in progesterone catabolism (50). However, in the bovine corpus luteum, PGF$_{2a}$ does not seem to have a significant effect on the stimulation of 20a-hydroxy steroid dehydrogenase and it is likely that the PGF$_{2a}$-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$_{2a}$-activated signal transduction pathways responsible for decreased steroidogenesis as well as structural luteolysis. In this 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$_{2a}$ 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). 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 this study indicated the onset of apoptosis in the luteal tissue at 18 h post-PGF$_{2\alpha}$ treatment. To characterize changes leading to the initiation of apoptosis, we included two additional time points (4 and 12 h) earlier to 18 h 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 (6–10) by others with regard to the initiation of apoptosis in 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$_L$, and so on) or pro-apoptotic (Bax, Bad, and so on), 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-apoptotic 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 the results of this 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). An analysis of luteal tissue revealed hypophosphorylation of Bad as early as 4 h 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). Although 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, and so on. In this study, although the total form of PARP increased in expression at 18 h, the cleaved form of PARP was only found to be upregulated at 18 h following PGF$_{2\alpha}$ injection compared with levels in the corpus luteum of control animals, further confirming the 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. The involvement of caspase-3 in mediating apoptosis in the mammalian cells is well established (52). Rueda et al. (53) found an increase in the caspase-3 activity 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 4–12 h 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 18 h, particularly that of caspase-3, could not be explained by changes in the expression or localization of the mitochondrial Bcl-2 family members, suggesting the 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 increased at different time points post-PGF$_{2\alpha}$ treatment suggested a role for the Fas pathway in the regulation of luteal cell apoptosis. Furthermore, the increased expression of Fas and its ligand (FasL) at 18 h suggests that the apoptosis induced by PGF$_{2\alpha}$ might be mediated by the Fas/FasL pathway.

As a positive control for DNase activity, equal amounts (50 µg) of cytosolic protein extract prepared from the corpus luteum collected before and at 18 h after PGF$_{2\alpha}$ treatment were preincubated with the indicated amounts of GST-ICAD-L (lanes 4–13), and the DNase activity was determined with isolated nuclei (3 × 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 the 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 (in bp) are indicated on the left.
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 occurred. However, it has been reported that the 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 18 h post-PGF$_{2\alpha}$ treatment suggests the 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, Carambulao et al. (23) using induced multiple follicular growth and ovulation model reported that PGF$_{2\alpha}$ can induce the 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 probable because of the induction of FasL and Fas expression by PGF$_{2\alpha}$. Interestingly, the activation of extrinsic apoptotic signaling cascade was observed only at 18 h and this might provide an explanation for the induction in the caspase-3 activity at the 18 h time point because caspase-3 is a downstream target of caspase-8. However, it does not explain the amplification in the mitochondrial cascade observed at 18 h. In this regard, it has been proposed that activated caspase-8 cleaves Bid and that this form (tBid) can amplify the mitochondrial cascade by modulating the ratio of pro-apoptotic to anti-apoptotic members at the level of mitochondria (57). Indeed, the 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 18 h, providing possible explanation for amplification in the caspase-9 activity observed at the 18-h time point.

Because it was impractical 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, and 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 this 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. The levels were similar to those 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 cannot be ruled out because caspase-8 is also known to act downstream of many other receptors that induce cell death such as tumor necrosis factor-α 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 the presence of a Ca$^{2+}$/Mg$^{2+}$-dependent temperature-sensitive DNase maximally active at neutral pH conditions in the crude cytosolic extract prepared from luteal tissue collected at 18 h 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 the 18 h 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 co-workers (43). Caspase-activated DNase has been reported to be activated during apoptosis by caspase-3-dependent cleavage of its inhibitor (ICAD). The examination of luteal tissue lysates for ICAD by Western blotting revealed increased presence of a ~30-kDa ICAD-reactive product at 12 h that apparently reflects a partially processed intermediate observed by others (43, 47). The partially cleaved ICAD further decreased at 18 h, suggesting that degradation of ICAD at 18 h post-PGF$_{2\alpha}$ might be responsible for the activation of the caspase-activated
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DNase in the luteal tissue, resulting in the fragmentation of chromosomal DNA into a 180-bp ladder. We reasoned that, if this were to be true, the 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, the 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-CAD. Together, these data provide convincing evidence for the involvement of CAD in apoptosis associated with PGF$_{2\alpha}$-induced luteal tissue apoptosis.

Based on the findings in this study and studies by others, we propose a model for the PGF$_{2\alpha}$-induced apoptotic signaling pathways in the corpus luteum (Fig. 11). Prostaglandin F$_{2\alpha}$-induced apoptotic signaling in the corpus luteum involves the activation of stress-activated protein kinases such as JNK and p38 MAPK (7, 60) that have been shown to result in the increased expression of genes that are primary initiators of apoptotic signaling machinery, viz. Bax and Fas/FasL (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. The 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 (23) by the absence of luteal apoptosis in response to PGF$_{2\alpha}$ in caspase-3 knock-out mice. 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 then are 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 this 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.

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