Apoptosis During Spontaneous and Prostaglandin F$_{2\alpha}$-Induced Luteal Regression in the Buffalo Cow (Bubalus bubalis): Involvement of Mitogenic-Activated Protein Kinases

Vijay K. Yadav, Ranga R. Sudhagar, and R. Medhamurthy

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560012, India

ABSTRACT

The present study was conducted to evaluate whether the corpus luteum (CL) of the water buffalo (Bubalus bubalis) cow undergoes luteal regression by the process of apoptosis and to examine the involvement of mitogen-activated protein (MAP) kinases during prostaglandin (PG) F$_{2\alpha}$-induced luteolysis. Sections of CL from late in the estrous cycle, i.e., during spontaneous luteolysis, stained for 4',6'-diamidino-2-phenylindole revealed increased numbers of condensed nuclei, indicating cell death by apoptosis, which was confirmed further by the occurrence of pronounced oligonucleosome formation. For morphological and biochemical characterization during PGF$_{2\alpha}$-induced apoptosis, CL were collected at 0, 4, 12, and 18 h after injection of 750 µg of Tiaprost, a synthetic analogue of PGF$_{2\alpha}$ to midestrous buffalo cows. Serum progesterone concentrations fell within 4 h and decreased (P < 0.05) maximally by 18 h. Concomitant decreases (P < 0.05) in the levels of steroidogenic acute regulatory mRNA and protein were observed in CL during 12–18 h, with the more profound effect on mRNA levels. Quantitative analysis of the genomic DNA showed a >5-fold increase (P < 0.05) in the low molecular weight DNA fragments by 18 h postinjection. Immunoblot analysis of CL tissue lysates showed increased (P < 0.05) levels of phospho-Jun N-terminal kinase (JNK) 1 (4- to 14-fold during 4–18 h) and phospho-p38 (2- to 4-fold at 18 h). Immunohistochemical evaluation of CL sections revealed an increased nuclear localization of phospho-JNK after treatment. These findings demonstrate that the CL of the buffalo cow undergoes cell death by the process of apoptosis both during spontaneous and PGF$_{2\alpha}$-induced luteolysis and that MAP kinases are involved during PGF$_{2\alpha}$-mediated apoptosis in the CL.

INTRODUCTION

The corpus luteum (CL) is an ephemeral endocrine structure that develops from a preovulatory/Graafian follicle after ovulation. Through biosynthesis and secretion of progesterone (P$_4$), it plays a pivotal role in the control of reproduction in mammals [1–3]. Regression of CL in the absence of conception is obligatory for initiation of a new reproductive cycle to allow for reovulation and another chance for conception to occur. However, during a reproductive cycle in which conception occurs and implantation ensues, the prolongation of luteal function beyond its usual life span is obligatory for maintenance of pregnancy [1, 2]. In a number of farm animals, including the water buffalo (Bubalus bubalis) cow, prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) is recognized as the physiological luteolysin that is responsible for regression (luteolysis) of CL at the end of a nonfertile cycle [3–5]. Despite the central role of PGF$_{2\alpha}$ in luteolysis, actual mechanisms during spontaneous luteolysis at the end of a nonfertile cycle or luteolysis that occur following exogenously administered PGF$_{2\alpha}$ are poorly defined. However, it is now well established that apoptosis or programmed cell death plays a central role in the regression of CL during PGF$_{2\alpha}$-induced or spontaneous luteolysis of several species including cattle [6–10].

PGF$_{2\alpha}$ acts on the CL by binding to a specific receptor belonging to the family of G protein-coupled receptors (GPCRs) localized mainly to large luteal cells but also present on small luteal and endothelial cells of the CL [5]. Upon binding to its receptor, PGF$_{2\alpha}$ induces activation of membrane-bound phospholipase-C, which catalyzes the hydrolysis of phosphotidyl inositol 4,5 bisphosphate to inositol trisphosphate (IP3) and diacyl glycerol (DAG) [11]. PGF$_{2\alpha}$-increased IP3 levels stimulate mobilization of intracellular Ca$^{2+}$, and increased DAG stimulates the Ca$^{2+}$-dependent protein kinase C (PKC) [12]. Although many of the anti-steroidogenic actions of PGF$_{2\alpha}$ in large luteal cells appear to be mediated by PKC [13, 14], stimulation of PKC by pharmacological agents under in vitro conditions does not induce cell death even though steroidogenesis is decreased [14]. These findings suggest that PGF$_{2\alpha}$ has additional effects and that the signaling pathways for steroidogenesis and cell death may be different in luteal cells.

Decreased steroidogenesis that occurs following PGF$_{2\alpha}$ administration in vivo and in vitro appears to be due to decreased transport of cholesterol to the inner mitochondrial membrane [15]. A mitochondrial protein, steroidogenic acute regulatory protein (StAR), plays a pivotal role in the transfer of cholesterol to the inner mitochondrial membrane, and this transfer is the proposed rate-limiting step in steroidogenesis [15]. Although StAR expression under experimental conditions of acute stimulation of steroidogenesis has been well studied [16], the effect of steroid synthesis inhibition that occurs during spontaneous or PGF$_{2\alpha}$-induced luteolysis is less understood.

Among the major types of signal transduction pathways in eukaryotic cells are protein kinase cascades that culminate in activation of protein kinases or mitogen-activated protein (MAP) kinases. In mammals, three major groups of MAP kinases have been identified: extracellular response kinase (ERK), Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK/p38). Each of these kinases is activated by a protein kinase cascade [17]. Recent work has revealed that activation of a number of
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FIG. 1. Serum concentrations of P4 (mean ± SEM) in water buffalo cows before and after Tiaprost (synthetic analogue of PGF2α) injection. Bars with different letters are significantly different (P < 0.05).

FIG. 2. StAR mRNA expression and ethidium bromide stained 28S and 18S rRNA bands (A) and StAR 30-kDa protein levels (B) in CL tissues retrieved from water buffalo cows before and after PGF2α analogue injection. Bars with different letters are significantly different (P < 0.05).

GPCRs by their respective ligands such as GnRH, thyrotropin-releasing hormone, and oxytocin also activates MAP kinases [18–20]. However, the nature of the biochemical pathways linking GPCRs to MAP kinases remains the subject of intense investigation. Because prostaglandins, including PGF2α, mediate their actions by activating specific GPCRs, it would be of interest to evaluate the role of MAP kinases during the process of luteolysis.

In the present study, experiments were conducted to determine the temporal course of biochemical and morphological changes associated with PGF2α-induced luteolysis in the water buffalo cow. Additionally, we sought to examine the role of MAP kinases during PGF2α-induced luteolysis. Our results indicate that luteal regression occurs by way of apoptosis in the buffalo cow. We also demonstrated activation of MAP kinases during PGF2α-induced luteolysis.

MATERIALS AND METHODS

Animals

All procedures in animals were approved by the Institutional Animal Ethics Committee, Indian Institute of Science. Water buffalo cows (Surthi breed) with a known history of normal cyclicity were recruited for the study. The day of onset of estrus was designated as Day 1 of the estrous cycle. Blood samples were obtained from animals selected for the study on Days 6–8 of the estrous cycle for determining the luteal phase P4 concentrations (2–4 ng/ml) to confirm the presence of a functional CL. Four readily identifiable changes in appearance of CL (stages I–IV) during a bovine estrous cycle have previously been reported [21, 22]. In the buffalo cow, the morphology of CL during the estrous cycle appears to be very similar to that in cattle. However, although the color of the CL apex is reddish as in cattle, the remainder of the CL body is also reddish rather than yellowish. Also, the stage IV CL is pale red to white in its appearance unlike the orange to yellow color in cattle. In this study, CL from stage II of the estrous cycle (midestrus) and stage IV (late estrus), i.e., during spontaneous luteolysis, were used.

CL Collection

Buffalo cows on Day 11 of the estrous cycle (CL at stage II) were injected i.m. with 750 µg of Tiaprost (Iliren; Intervet International, Boxmeer, Holland), a synthetic analogue of PGF2α, (three or four animals/time point). At 4, 12, and 18 h following PGF2α injection, ovaries were collected into cold PBS and washed in PBS prior to processing. Under sterile conditions, the CL from the ovary was extirpated, cut into six to eight pieces, transferred to labeled cryovials, snap frozen in liquid nitrogen, and stored at −70°C until analysis. Also, a small portion of CL tissue was fixed in Bouin solution for histological examination. The processing of CL was completed within 30 min after collection. Blood samples were collected immediately prior to PGF2α injection and at the time of collection of ovaries. Serum was stored at −20°C until assayed for P4 concentrations. Stage II and stage IV CL (designated as control [0 h] and spontaneous luteolysis [SL], respectively) were collected from untreated buffalo cows at a nearby slaughterhouse according to the morphological criteria established for CL classification in cattle [21, 22]. Blood samples were collected 1 day prior to slaughter for assaying serum P4 concentrations, and CL were from buffalo cows that had P4 concentrations >2 ng/ml (stage II) or <2 ng/ml (stage IV).

Reagents

The polyclonal antibodies specific to phospho-p38 MAPK (9211), phospho-SAPK/JNK (9251), phospho-p42/44 MAPK (9101S), p38 MAPK (9212), ERK1 (sc-19), ERK2 (sc-154), JNK1 (sc-571), and JNK2 (sc-572) were purchased from Cell Signaling Technology (Beverly, MA; 9101S, 9211, 9212, 9251) and Santa Cruz Biotechnology (Santa Cruz, CA; sc-
FIG. 3. A) Representative hematoxylin and eosin-stained section of CL retrieved from control (0 h) and PGF$_{2\alpha}$ analogue-treated (4, 12, and 18 h) water buffalo cows. Note the appearance of morphologically different cells at 18 h posttreatment. Solid arrowheads indicate nuclei of abnormal cells. ×40. B) DAPI-stained control CL tissue (0 h), CL tissue 18 h after PGF$_{2\alpha}$ analogue injection, and stage IV CL tissue (spontaneous luteolysis; SL). Open arrowheads indicate apoptotic nuclei. Bars with different letters indicate significant differences (P < 0.05). ×100, zoom 2.6.

19, sc-154, sc-571, sc-572). Polyclonal antibodies against StAR were a gift from Professor D.M. Stocco (Texas Tech University Health Sciences Center, Lubbock, TX).

Terminal deoxynucleotidyl transferase was purchased from Amersham Biosciences (Asia Pacific, Hong Kong). Genescreen Plus and polyvinylidene fluoride (PVDF) membranes were purchased from NEN Life Sciences (Boston, MA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Gibco BRL (Gaithersburg, MD) or were obtained locally.

Histology

CL tissue fixed in Bouin solution was dehydrated through a graded series of ethanol solutions, cleared in xylene, embedded in paraffin, and sectioned at 3- to 4-μm thickness. After rehydration through a series of graded alcohol solutions to PBS, the sections were mounted on slides, stained with hematoxylin and eosin, and observed and photographed by light microscopy.

4′,6′-Diamidino-2-Phenylindole Staining

Ovaries containing CL were removed after slaughter and transported in PBS on ice to the laboratory. Under sterile conditions, the CL was extirpated, put on cryotome holders, and stored at −20°C until sectioning. Approximately 5-μm-thick cryosections were taken using a Cryotome (Reichert Jung, Arnsberg, Germany). The sections were permeabilized with 1% Triton X-100 in PBS for 4 min at room temperature and then stained with 4′,6-diamidino-2-phenylindole (DAPI; 100 μg/ml) in PBS for 10 min at room temperature. Slides were washed twice with PBS for 5 min each time, mounted in glycerol, and observed under a confocal microscope (Leica TCS, Wetzlar, Germany).

Immunohistochemistry

CL were fixed for 24–48 h in Bouin fluid, dehydrated through a graded series of ethanol solutions, cleared in xylene, and embedded in paraffin. Approximately 5-μm-thick sections were cut, deparaffinized, and blocked in 5% normal goat serum (in 0.5% BSA-PBS) at 37°C for 30 min. Sections were incubated with the primary antibody, polyclonal phospho-JNK (1:100 in 0.5% BSA-PBS) at 37°C for 1 h, followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:16 in 0.5% BSA-PBS) at 37°C for 30 min. After extensive washes, sections were mounted in glycerol and visualized under a confocal microscope.

Isolation of Genomic DNA and Analysis

Genomic DNA was extracted from individual CL, precipitated, dissolved in distilled water, and spectrophotometrically quantitated as described previously for DNA fragmentation analysis [23–25]. Genomic DNA (30 μg for agarose electrophoresis or 1 μg for quantitative analysis) was labeled at the 3′ end with 50 μCi of [α-32P]dCTP (3000 Ci/mM; NEN Life Sciences) by incubation in a 37°C water bath for 60 min with 25 IU of terminal transferase. The reaction was terminated using 5 μl of 0.5 M EDTA followed by incubation at 70°C for 2–3 min. The DNA samples were resolved on a 2% agarose gel. The resolved DNA was transferred onto a nitrocellulose filter using a capillary transfer blotting apparatus. The membrane was washed with 5× saline sodium citrate (SSC) buffer for 3 min to remove the free label. The membrane was covered with plastic wrap and exposed to Ko-
RNA Extraction and Northern Hybridization

Total RNA was extracted from CL tissue using Trizol reagent according to the manufacturer’s recommendations. Equal amounts of RNA (20 μg) from each sample, quantitated by absorbance at 260 nm, were subjected to electrophoresis in a 1% agarose-formaldehyde gel and subsequently transferred to nylon membranes (Genescreen Plus). The blot was then hybridized to a mouse StAR cDNA probe labeled using a random primer labeling kit (NEN Life Sciences). Hybridization was carried out in Church buffer [27] at 60°C for 16 h, and washed to a stringency of 0.2× SSC plus 0.1% SDS at 60°C, followed by autoradiography with an intensifying screen at ~70°C. Hybridization signal was also quantified using a PhosphorImager (BAS 1800; Fuji). RNA integrity and equal loading was visualized by examination of 18S and 28S rRNA after ethidium bromide staining of DNA on agarose gels.

Preparation of Tissue Lysates for Immunoblotting

CL tissue lysate was prepared following the previously published procedures with some modifications [28]. Frozen CL tissue was ground to powder, transferred to Eppendorf tubes containing 200–500 μl of RIPA buffer (10 mM NaPO₄, pH 7.0, 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 50 mM NaF, 200 mM Na₃VO₄, 0.1% β-mercaptoethanol, 1 mM PMSF, 4 μg/ml aprotinin, and 2 μg/ml leupeptin), and incubated on ice for 30 min with intermittent mixing before centrifugation at 15,000 × g for 10 min at 4°C. The clarified lysate was recovered, aliquoted, and stored at −70°C. An aliquot was used for protein estimation by the Bradford (microassay) method [29].

Western Blot Analysis

CL tissue lysate (100 μg protein) was resolved by 10% or 12% SDS-PAGE and electrophoretically transferred onto PVDF membrane using a semidyide transfer unit (Bio-Rad Laboratories, Richmond, CA). Nonspecific sites on the membrane were blocked using 10% milk in TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) by incubating overnight at 4°C. The membrane was then washed extensively in 1× TBST (three times for 5 min each at room temperature) and incubated at room temperature with primary antibody specific for different proteins (1:250 to 1:1000 in TBST containing 0.2% BSA) for 3 h at room temperature. At the end of the incubation, the membrane was washed and incubated with secondary antibody (horseradish peroxidase labeled anti-rabbit IgG) at 1:2500 dilution in TBST containing 5% milk. The bands were then visualized using an ECL kit (NEN Life Sciences). Autoradiographs were scanned using a UVI-Tech gel documentation system and quantitated using UVI-Band Map (1999) software.

Effect of PGF₂α on Serum P₄ and StAR mRNA and Protein Levels

Circulating serum P₄ concentrations prior to PGF₂α analogue injection were not different in untreated and treated animals. Bar graph (D) represents the quantitative measurement of low molecular weight DNA labeling as a percentage of difference from the control (time 0 h). Bars with different letters above them are significantly different (P < 0.05).

Progestosterone Assay

Progestrone concentrations in serum were determined by a specific RIA as reported previously [30]. The sensitivity of the assay was 0.1 ng/ml and the inter- and intraassay coefficients of variation were <10%.

Statistical Analyses

Wherever applicable, data were 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 a Tukey multiple comparison test (PRISM Graph Pad version 2; Graph Pad Software, San Diego, CA). Differences at P < 0.05 were considered significant.

RESULTS

FIG. 4. Genomic DNA isolated from CL obtained from control animals (stage II of estrous cycle), during spontaneous luteolysis (SL, stage IV of estrous cycle), and at different time points after PGF₂α analogue injection. DNA was subjected to either qualitative (A and B) or quantitative (C) analysis. Agarose gel electrophoresis and ethidium bromide staining of DNA obtained from SL clearly show DNA ladder ing in contrast to lack of DNA laddering in DNA from control animals. Bar graph (D) represents the quantitative measurement of low molecular weight DNA labeling as a percentage of difference from the control (time 0 h). Bars with different letters above them are significantly different (P < 0.05).

FIG. 5. Immunoblot analysis of phospho-p38 (p-p38) and total p38 (p38) before and after PGF₂α treatment. CL tissue lysates (100 μg) were resolved by SDS-PAGE, transferred to PVDF membranes, probed with p-p38, stripped, and reprobed with p38 antibody. The blots shown are from one of three independent experiments (CL from one animal from each time point). The arbitrary densitometric units from time 0 h were set as 100%, and results at other time points are expressed in relation to the 0 h value.
animals; therefore, the data were combined and presented as the time 0 h value (Fig. 1). The mean serum P₄ concentrations were 2.70 ± 0.18 ng/ml at 0 h. The concentrations were decreased ($P < 0.05$) by 4 h postinjection, and at 18 h they were 0.68 ± 0.06 ng/ml, significantly less ($P < 0.05$) than the 0 h value (Fig. 1).

The mouse StAR cDNA probe [26] hybridized with two species of RNA from the CL of the buffalo cow, one major band (~2.9 kilobases [kb]) and one minor band (~1.6 kb) (Fig. 2A). No hybridization to StAR cDNA was observed for RNA isolated from the muscle tissue (data not shown). Examination of StAR expression in CL tissues obtained from control and PGF₂α-analogue-treated animals revealed that the mRNA levels (2.9-kb band) tended to be lower ($P > 0.05$) within 4 h after injection and were significantly ($P < 0.05$) decreased, to 45% and 16% of the 0-h value, at 12 and 18 h postinjection, respectively (Fig. 2A). Western blot analysis of CL tissue lysates revealed that the StAR protein (30 kDa) levels were <50% those of control levels at both 12 and 18 h postinjection ($P < 0.05$; Fig. 2B), indicating that mRNA and protein levels of StAR decreased within 12 h postinjection.

**Morphological and Biochemical Analyses of Apoptosis**

Typical hematoxylin and eosin-stained sections of CL tissues retrieved at different times (three samples per time point) following PGF₂α-analogue injection are presented in Figure 3A. Histological evaluation of sections collected at 18 h postinjection revealed a number of morphologically abnormal cells with condensed nuclei (Fig. 3A). The sections collected at 0, 4, and 12 h postinjection did not show marked changes (Fig. 3A). These observations were confirmed with an analysis of in situ apoptosis by identifying the apoptotic cells using DAPI staining. To estimate the percentage of cell death, CL sections (three per time point) from control, 18 h postinjection, and SL were stained with DAPI. The apoptotic nuclei identified by DAPI staining were condensed and small compared with the large nuclei in the normal cells (Fig. 3B). The proportion of dead cells was higher than 40% ($P < 0.05$) in sections of CL obtained 18 h postinjection and during spontaneous regression (Fig. 3B).

We next examined the biochemical DNA integrity of CL collected from untreated animals during stages II and IV of the estrous cycle ($n = 3$) to determine DNA laddering, considered a hallmark of apoptosis. Agarose gel electrophoresis and ethidium bromide staining of genomic DNA isolated from CL during stage IV, i.e., during spontaneous regression, showed the characteristic pattern of DNA laddering indicative of apoptotic cell death in the luteal tissue (Fig. 4A). However, DNA isolated from CL collected from stage II did not show laddering (Fig. 4A). When genomic DNA isolated from CL before PGF₂α-analogue injection and at various time points (three per time point) postinjection was analyzed for low molecular weight fragments, the results indicated that DNA fragments could be visualized consistently at 18 h posttreatment (Fig. 4B). Quantitation of DNA fragments indicated that the low molecular weight DNA labeling increased ($P < 0.05$) >500% at 18 h compared with that observed in CL collected from control animals (Fig. 4, C and D). Low molecular weight DNA labeling did not increase in genomic DNA isolated from CL of buffalo cows at 4 and 12 h postinjection (Fig. 4D).

**Involvement of MAP Kinases During PGF₂α-Induced Apoptosis**

Figure 5 shows representative immunoblots and integrated arbitrary densitometric unit data of phospho-p38 and total p38 MAPK levels (expressed as a percentage of the results obtained at 0 h) from CL collected at different time points after PGF₂α-analogue treatment. Phospho-p38 levels increased ($P < 0.05$) 2- to 4-fold higher ($P < 0.05$) at 4 and 12 h postinjection compared with the CL collected from the control animals, and the levels were ~2- to 4-fold higher ($P < 0.05$) at 18 h. Total p38 levels tended to be higher at 18 h but were not significantly increased at the time points tested (Fig. 5).

Immunoblot analyses of phosphorylation-dependent (p-) and -independent (total) levels of JNK1 and JNK2 are presented in Figure 6. The p-JNK1 levels increased ($P < 0.05$) 4-fold at 4 and 12 h after PGF₂α-analogue injection compared with the level at 0 h and increased further ($P < 0.05$), ~14-fold, by 18 h postinjection (Fig. 6A). However, JNK1 levels did not change significantly from levels found at 0 h (Fig. 6A). Similarly, although the p-JNK2 from CL collected at different time points postinjection tended to be higher ($P = 0.08$), the levels were not significantly different from those observed in CL of control animals (Fig. 6B). Phosphorylation-independent levels were not significantly different ($P < 0.05$) for the control and treated animals (Fig. 6B).

We then examined the immunohistochemical staining for p-JNK using an antibody that recognizes both the p-JNK1 and p-JNK2 in sections of CL from 0 and 18 h postinjection. Typical sections are presented in Figure 7. Consistent...
with the increased p-JNK1 and to a lesser extent p-JNK2 levels, nuclear staining of p-JNK increased progressively starting at 4 h and was highest at 18 h postinjection, the maximum posttreatment observation point in the present study. The immunohistochemistry findings confirm the immunoblot data of profound increase in p-JNK levels during PGF2α-induced apoptosis and further suggests that the p-JNK levels are localized mainly in the nuclei after PGF2α analogue injection.

The total ERK1 (44 kDa), ERK2 (42 kDa), and phospho-ERK1/2 levels were evaluated for their involvement during PGF2α-induced apoptosis, and the results are presented in Figure 8. The total ERK1, ERK2, and phospho-ERK1/2 levels in CL lysates obtained at different time intervals after PGF2α analogue injection did not differ from the levels observed in CL lysates obtained from untreated animals.

DISCUSSION

To date, the nature of luteal regression has not been studied in the water buffalo cow. Our findings in the present study demonstrate for the first time in this species that the spontaneously regressing CL exhibits apoptosis, as reflected by internucleosomal DNA fragmentation and formation of the typical DNA ladder, a clear biochemical hallmark of apoptosis. A number of associated endocrine and morphological changes noted in the buffalo cow are quite similar to those in cattle [4]. We recently quantitated PGF2α receptors in the CL of the buffalo cow [31], and the affinity and number of receptors observed are quite similar to those reported during different stages of the estrous cycle in cattle [32]. It is not surprising that exogenous administration of PGF2α also induced luteolysis by the process of apoptosis during midestrous cycle in the buffalo cows. The P4 profile and the time course of appearance of DNA laddering are strikingly similar to the PGF2α-induced luteolysis reported previously for cattle and sheep [6–8].

To determine the role of StAR during PGF2α-mediated suppression of steroidogenesis, we examined the mRNA expression and protein levels in the CL obtained from control and PGF2α-treated buffalo cows. Our results are consistent with the findings of others who have studied StAR expression and protein levels after induction of luteolysis in a number of species [33–36]. Pescador et al. [33] studied mRNA expression and protein levels in the CL of the cattle and observed a 50% decline in mRNA within 12 h of PGF2α injection, and mRNA levels were undetectable by 24 h postinjection. In the ewe, Juengel et al. [34] reported that even though serum P4 levels declined within 4 h after PGF2α injection, the decline in StAR mRNA levels were apparent only at 12 h postinjection. Recently, Tsai et al. [35] reported that StAR mRNA in the bovine CL declined as early as 4 h after PGF2α injection. The pattern of decrease in StAR protein observed in the present study appears to be similar to the time course of decrease in StAR protein levels in the CL during GnRH-induced luteolysis in women [36]. Unlike the dramatic fall in the levels of mRNA, the levels of protein declined only to ~50% of control levels, suggesting that the mature 30-kDa protein appears to decline slowly compared with other forms of StAR. The time course of decrease in StAR protein after PGF2α injection noted in the present study is at variance with other findings of decreased levels within 1 h after PGF2α injection in the rat CL [37]. Although the present study was not designed to address the site and mechanism of action of PGF2α on StAR, recent studies indicate that gonadotropins and growth hormone appear to enhance mRNA levels, whereas PGF2α suppresses both basal and gonadotropin-stimulated mRNA levels [38, 39]. Juengel et al. [38] reported that PGF2α treatment decreased mRNA levels apparently by activating PKC. However, involvement of SF-1 and the negative transcription factor DAX-1 in mediating the StAR gene transcription has been suggested.

FIG. 7. Representative sections of immunohistochemical staining for phospho-JNK. The CL was collected from water buffalo cows at 0, 4, 12, and 18 h after PGF2α analogue injection. Phospho-JNK was detected using p-JNK antibody with FITC-conjugated goat anti-rabbit antibody. ×100.

FIG. 8. Immunoblot analysis of phospho-ERK1/2 and total ERK1/2 before and after PGF2α analogue treatment. This blot is from one of three independent experiments (CL from one animal from each time point). The arbitrary densitometric units from time 0 h were set as 100%, and the results at other time points posttreatment are expressed in relation to the 0 h value.
Recently, repression of StAR transcription by enhanced DAX-1 levels following PGF$_2$$_a$ treatment has been reported in rats [39]. Because StAR has three phosphorylation sites for PKC, activation of PKC by PGF$_2$$_a$, is expected to decrease and/or block the cholesterol transport and consequently decrease P$_4$ biosynthesis [3]. However, there is as yet no experimental evidence to support this hypothesis.

The morphological and biochemical data on spontaneous and PGF$_2$$_{a}$-induced luteolysis reported in the present study are similar to findings reported by others for cattle [6, 7] and sheep [8]. Apoptosis is a multistep process regulated by the interplay of a multitude of survival and apoptotic factors that participate in the process to ultimately decide whether a cell survives or become apoptotic. Although DNA degradation in apoptotic cells is regarded as part of the executionary phase of apoptosis (i.e., the final events leading to cell death), the events leading to apoptosis during luteolysis are still not well characterized and are the subject of intense investigation. Despite intensive efforts, the intracellular signaling pathways that mediate apoptosis also remain poorly characterized. Many extracellular stimuli are converted into specific cellular responses through the activation of MAP kinase signaling pathways. Recent studies indicate that the critical pathways related to cell survival, growth, and apoptosis are regulated by distinct MAP kinase signaling modules [17]. Although the ERK signaling module (activated by mitogenic stimuli) plays a critical role in cell proliferation, the roles of JNK and p38 MAPK signaling modules (activated by nonmitotic stimuli) are only now beginning to be understood. In the present study, we demonstrated for the first time under in vivo conditions activation of JNK and p38 MAPK signaling modules in the CL in response to PGF$_2$$_a$ injection. Using in vitro cell culture systems involving transformed cell lines, several other investigators have demonstrated that stress stimuli such as ultraviolet light, chemicals, shear, and heat couple these modules to either receptor tyrosine kinases or G proteins [40–43]. Chen et al. [44] were the first to report stimulation of ERK signaling module by the PGF$_2$$_a$ in bovine luteal cells under in vitro conditions. Although the importance of activation of the ERK signaling pathway by PGF$_2$$_a$, or by pharmacological agents that activate PKC in the luteal cells remains to be determined, the observation of Chen et al. [44] demonstrated the role of MAP kinase cascades in gene transcription in the luteal tissue. Rueda et al. [45] demonstrated that exposure of bovine luteal cells in vitro to ultraviolet irradiation led to activation of phosphorylated forms of JNK and p38 MAPK and attenuation of ERK, providing evidence for an important role for intracellular MAP kinase signaling pathways during luteal function and regression. Except for the lack of an effect of PGF$_2$$_a$ on ERK levels, the in vivo findings of the present study appear strikingly similar to the findings reported by Rueda et al. [45]. These findings suggest that luteal cells could be used under in vitro conditions to study PGF$_2$$_a$-induced signaling mechanisms responsible for the initiation of apoptosis; earlier studies did not reveal any correlation between PGF$_2$$_a$ treatment and apoptosis. In the present study, it is not clear whether activated p38 and JNK signaling modules are responsible for the PGF$_2$$_a$-induced apoptosis, and more studies are required to find a correlation between the two events. However, levels of phosphorylated forms of p38 and JNK during the first 4–12 h after PGF$_2$$_a$ injection were either similar to control levels or were slightly elevated, but a dramatic increase, especially of p-JNK1, was observed at 18 h postinjection. This pattern makes it difficult to say whether these events are causally related to apoptosis, because DNA laddering (considered as execution phase of apoptosis) was consistently observed also at 18 h postinjection. Nonetheless, the observation that a luteolytic dose of PGF$_2$$_a$ activates JNK and p38 signaling modules indicates that these kinases may play an important role in the PGF$_2$$_a$-mediated apoptotic cell death in the CL. The critical role of MAP kinases in apoptotic cell death has been well established from numerous studies carried out using a variety of cell lines [46–48]. Additionally, the transient transfection studies using activated mutants of MAP kinases also have confirmed the importance of the JNK pathway in the apoptosis of cells [49].

We demonstrated that the CL of the water buffalo cow undergoes regression by apoptosis. Furthermore, the results of this study suggest an important role for MAP kinases in the initiation of onset of PGF$_2$$_a$-mediated apoptosis in the CL. It will be necessary to delineate the downstream signaling MAP kinase pathways to elucidate the mechanism(s) involved during luteolysis.

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