

Apoptosis During Spontaneous and Prostaglandin F_{2α}-Induced Luteal Regression in the Buffalo Cow (*Bubalus bubalis*): Involvement of Mitogen-Activated Protein Kinases

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ABSTRACT

The present study was conducted to evaluate whether corpus luteum (CL) of the buffalo cow undergoes luteal regression by the process of apoptosis, and to examine the involvement of mitogen-activated protein (MAP) kinases during PGF_{2α}-induced luteolysis. Sections of CL from late-estrous cycle i.e., during spontaneous luteolysis, stained for DAPI revealed increased number of condensed nuclei indicating cell death by apoptosis confirmed further by the occurrence of pronounced oligonucleosome formation. For morphological and biochemical characterization during PGF_{2α}-induced apoptosis, CL were collected at 0, 4, 12 and 18 h after injection of 750 µg of Tiaprost, a synthetic analog of PGF_{2α} to mid-estrous cycle buffalo cows. Serum P₄ concentrations fell within 4 h and decreased (p < 0.05) maximally by 18 h. A concomitant decrease (p < 0.05) in the levels of StAR mRNA and protein were observed in CL during 12-18 h, with 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 post injection. Immunoblot analysis of CL tissue lysates showed increased (p < 0.05) levels of phospho-JNK1 (4-14 fold during 4-18 h) and phospho-p38 (2-4 fold at 18 h). Immunohistochemistry of CL sections revealed an increased nuclear localization of

phospho-JNK after treatment. These findings demonstrate that CL of the buffalo cow undergoes cell death by the process of apoptosis both during spontaneous and PGF_{2α}-induced luteolysis, and that MAP kinases are involved during PGF_{2α}-mediated apoptosis in the CL.

INTRODUCTION

Corpus luteum (CL) is an ephemeral endocrine structure that develops from a preovulatory/Graafian follicle after ovulation. Through biosynthesis and secretion of progesterone (P₄), 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. On the other hand, during a reproductive cycle in which conception occurs and implantation ensues the prolongation of luteal function beyond its life span is obligatory for maintenance of pregnancy [1, 2]. In a number of farm animals including the buffalo cow, prostaglandin F_{2α} (PGF_{2α}) is recognized as the physiological luteolysin that is responsible for regression (luteolysis) of CL at the end of a non-fertile cycle [3-5]. Despite the central role of PGF_{2α} in luteolysis, actual mechanisms during spontaneous luteolysis at the end of a non-fertile cycle or luteolysis that occur following exogenously administered PGF_{2α} 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_{2α}-induced or spontaneous luteolysis of several species including the cattle [6-10].

Prostaglandin F_{2α} 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 is also present on small luteal and endothelial cells of the CL [5]. Upon binding to its receptor, $\text{PGF}_{2\alpha}$ has been reported to induce activation of membrane-bound phospholipase-C that catalyzes the hydrolysis of phosphatidyl inositol 4,5 bisphosphate to inositol trisphosphate (IP3) and diacyl glycerol (DAG) [11]. Prostaglandin $\text{F}_{2\alpha}$ -increased IP3 levels have been reported to stimulate mobilization of intracellular Ca^{2+} , and increased DAG is reported to stimulate the Ca^{2+} -dependent protein kinase C (PKC) [12]. Although many of the anti-steroidogenic actions of $\text{PGF}_{2\alpha}$ in large luteal cells appear to be mediated by PKC [13,14], but stimulation of PKC by pharmacological agents under in vitro conditions does not induce cell death even though they bring about decreased steroidogenesis [14] suggesting that $\text{PGF}_{2\alpha}$ has additional effects, and that the signaling pathways for steroidogenesis and cell death may be different in luteal cells.

Decreased steroidogenesis that occur following $\text{PGF}_{2\alpha}$ in vivo and in vitro conditions appears to be due to decreased transport of cholesterol to the inner mitochondrial membrane [15]. In this regard, a mitochondrial protein i.e., steroid acute regulatory protein (StAR), plays a pivotal role in the transfer of cholesterol to the inner mitochondrial membrane, 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 occur during spontaneous or $\text{PGF}_{2\alpha}$ -induced luteolysis is less well studied.

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, and each of these kinases is activated by protein kinase cascade [17].

They are extracellular response kinase (ERK), Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK/p38). Recently, it has been shown that activation of a number of GPCRs by their respective ligands such as gonadotropin releasing hormone, thyrotropin releasing hormone and oxytocin also activate MAP kinases [18-20]. However, the nature of biochemical pathways linking GPCRs to MAP kinases remains the subject of intense investigation. Since prostaglandins including the $\text{PGF}_{2\alpha}$ 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, therefore, experiments were carried out to determine the temporal course of biochemical and morphological changes associated with the $\text{PGF}_{2\alpha}$ -induced luteolysis in the buffalo cow. Additionally, we sought to examine the role of MAP kinases during the $\text{PGF}_{2\alpha}$ -induced luteolysis. Our results indicate that luteal regression occurs by way of apoptosis in the buffalo cow. Furthermore, we demonstrate activation of MAP kinases during $\text{PGF}_{2\alpha}$ -induced luteolysis.

MATERIALS AND METHODS

Animals

All procedures in animals were approved by the Institutional Animal Ethics Committee, Indian Institute of Science. Buffalo cows (*Bubalus bubalis*; 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. Animals selected for the study (treatment groups, see below) were subjected to blood sampling on day 6-8 of the estrous cycle for determining the luteal phase P_4 concentrations (2-4 ng/ml) to confirm the

presence of functional CL. Four readily identifiable changes in appearance of CL (Stage I to IV) during a bovine estrous cycle has previously been reported [21,22]. In the buffalo cow, the morphology of CL during estrous cycle also appears to be very similar to the cattle. However, it should be pointed out that while the color of apex of CL is reddish just as in the cattle, but the remainder of the body of the CL is also reddish and not yellowish in its appearance. Also, the Stage IV of estrous cycle is pale red to white in its appearance unlike the orange to yellow color in the cattle. In this study, CL from Stage II of estrous cycle corresponding to mid-estrous cycle and Stage IV of estrous cycle corresponding to late-estrous cycle i.e., during spontaneous luteolysis have been used (see below).

Corpus Luteum Collection

Buffalo cows on day 11 of the estrous cycle (corresponding to Stage II of estrous cycle) were injected with 750 µg of Tiaprost (Iliren^R) i.m., a synthetic analog of PGF_{2α} (n = 3-4 animals/time point). At 4, 12 and 18 h following PGF_{2α} 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 6-8 pieces, transferred to labeled cryovials, snap frozen in liquid nitrogen and stored at -70⁰C until analysis. Also, a small portion of CL tissue was fixed in Bouin's solution for histological examination. The processing of CL was completed within 30 min after killing. Blood samples from the buffalo cows were collected immediately prior to injection of PGF_{2α} and at the time of collection of ovaries. Serum was stored at -20⁰C until assayed for P₄ concentrations. Corpora lutea from untreated buffalo cows were collected from a nearby slaughterhouse. Corpora lutea of Stage II of estrous cycle were designated as time 0 h collection and

Stage IV of estrous cycle corresponding to the late estrous cycle i.e., during the period of expected spontaneous luteolysis (SL), were collected from the slaughterhouse according to the morphological criteria established for classification of CL during different stages of the estrous cycle in the cattle by Ireland et al. [21] and Okuda et al. [22]. Blood samples were collected one day prior to slaughter for assaying serum P₄ concentrations, and the CL was included in the study 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 (# 9100), p38 MAPK (# 9212), ERK1 (# sc-19), ERK2 (# sc-154), JNK1 (# sc-571) and JNK2 (# sc-572) were purchased from Cell Signaling Technology, Beverly, MA (# 9100, # 9211, # 9212, # 9251) and Santa Cruz Biotechnology Inc., Santa Cruz, CA (# sc-19, # sc-154, # sc-571 and # sc-572). Polyclonal antibodies against StAR were a kind gift from Professor D M Stocco of Texas Tech University Health Sciences Center, Texas, USA.

Terminal deoxy-nucleotidyl transferase (tdt) was purchased from Amersham Pharmacia. Genescreen Plus and PVDF membranes were purchased from NEN Life Sciences, Boston, MA. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO, Gibco BRL, Gaithersburg, MD, or sourced locally.

Histology

Corpus luteum tissue fixed in the Bouin's solution was dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin and sectioned (3-4 µm thickness). The sections mounted on slides, after rehydration through a series of graded

alcohol to PBS, were stained with hematoxylin and eosin, observed and photographed by light microscopy.

4', 6'-Diamidino-2-Phenylindole (DAPI) Staining:

Ovaries containing CL was 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 Cryotome (Reichert Jung, Germany), and the sections were stained with DAPI (100 $\mu\text{g}/\text{ml}$) in PBS for 10 min at room temperature following permeabilization with 1% Triton X-100 in PBS for 4 min at room temperature. Slides were washed with PBS for 5 min 2X, mounted in glycerol and observed under Confocal microscope (Leica TCS, Germany).

Immunohistochemistry

Corpora lutea were fixed for 24-48 h in Bouin's fluid, dehydrated through a graded series of ethanol, cleared in xylene and embedded in paraffin. Approximately 5 μm thick sections were 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 antibody (1:100 in 0.5% BSA-PBS) at 37°C for 1 h, followed by 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 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 either subjected to agarose gel electrophoresis, stained with ethidium bromide and DNA visualized by UV transillumination, or analyzed for quantitation of low molecular weight DNA fragments as described previously [24] with a few modifications. Briefly, 1 µg of genomic DNA was labeled at the 3'-end with 50 µCi of [α^{32} P]-deoxy ATP (3000 Ci/mM; NEN Life Sciences, Boston, MA) by incubating at 37⁰C 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 incubating the reaction tubes at 70⁰C for 2-3 min. The DNA samples were resolved on 2% agarose gel. The resolved DNA was transferred on to hybridization transfer membrane using the alkaline capillary blotting transfer method. The membrane was washed with 5X SSC buffer for 3 min to rid of the free label. The membrane was covered with Saran wrap and exposed to Kodak-O-MAT films at -70⁰C in an X-ray cassette for different time periods. Additionally, the membrane was exposed to phosphor imager (BAS-1800, Fuji films, Tokyo, Japan) to densitometrically quantify the low molecular weight labeled DNA.

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 the absorbance at 260 nm, were subjected to electrophoresis in 1% agarose-formaldehyde gel and subsequently transferred to Nylon membrane (Gene screen

plus). The blot was then hybridized to mouse StAR cDNA probe [26] labeled using random primer labeling kit (NEN, Life Sciences, Boston, MA). Hybridization was carried out in Church buffer [27] at 60°C for 16 h and washes were to a stringency of 0.2X SSC plus 0.1% SDS at 60°C, followed by autoradiography with an intensifying screen at –70°C. Additionally hybridization signal was quantified using a phosphorimager (BAS 1800, Fuji film, Tokyo, Japan). RNA integrity and equal loading was visualized by examination of 18S and 28S rRNA after ethidium bromide staining of formaldehyde gels.

Preparation of Tissue Lysates for Immunoblotting

Corpus luteum tissue lysate was prepared following the previously published procedures with some modifications [28]. Briefly, 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 using Bradford (micro-assay) method [29].

Western Blot Analysis

The CL tissue lysate (100 µg protein) was resolved by 10% or 12% SDS-PAGE and electroblotted onto PVDF membrane using semi-dry transfer unit (Bio-Rad Laboratories, Richmond, CA). Nonspecific sites on the membrane were blocked using 10% milk in TBST (20 mM TrisCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) by incubating for overnight at 4°C. The membrane was then washed extensively in 1X

TBST (3X, 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 ECL kit (NEN Life Sciences, Boston, MA). Autoradiographs were scanned using UVI-Tech gel documentation system and quantitated using UVI-Band Map (1999) software.

Progesterone Assay

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

Statistical Analyses

Data wherever applicable was expressed as mean \pm 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 Turkey's multiple comparison test (PRISM Graph Pad version 2, Graph Pad software Inc., San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of PGF_{2 α} on serum P₄, StAR mRNA and protein levels

Circulating serum P₄ concentrations prior to PGF_{2 α} analog injection between untreated and treated animals did not vary ($p > 0.05$) significantly, therefore, the data

were combined and presented as time 0 h value (Fig.1). The mean serum P₄ concentrations were 2.70 ± 0.18 ng/ml at 0 h, decreased ($p < 0.05$) by 4 h post injection and at 18 h, the concentrations were 0.68 ± 0.06 ng/ml, significantly ($p < 0.05$) less than that at the 0 h (Fig. 1).

As can be seen in Fig. 2A, the mouse StAR cDNA probe [26] observed to hybridize with two species, one major (~2.9 Kb) and one minor (~1.6 Kb) band, of RNA from the CL of the buffalo cow. No hybridization to StAR cDNA was observed to RNA isolated from the muscle tissue (data not shown). Examination of StAR expression in CL tissues obtained from control and PGF_{2 α} analog-treated animals revealed that the mRNA levels (2.9 Kb band) tended to be lower ($p > 0.05$) within 4 h after injection, and significantly ($p < 0.05$) decreased to 45% and 16% of time 0 h at 12 and 18 h post injection, respectively (Fig. 2A). Western blot analysis of CL tissue lysates revealed that the StAR protein (30 kDa) levels were <50% of control levels both at 12 and 18 h post PGF_{2 α} analog injection ($p < 0.05$, Fig. 2B) indicating that mRNA as well as protein levels of StAR decreased within 12 h post injection.

Morphological and Biochemical Analyses of Apoptosis

Typical hematoxylin and eosin stained sections of CL tissues retrieved at different time points following PGF_{2 α} analog injection are presented in Fig. 3A. Histological evaluation of section of CL tissues ($n = 3$) collected at 18 h post injection revealed a number of morphologically abnormal cells with condensed nuclei (Fig. 3A). The sections from CL tissues ($n = 3$) collected at 0, 4 and 12 h after PGF_{2 α} analog injection did not show marked changes (Fig. 3A). These observations were confirmed with in situ apoptosis by determining the apoptotic cells using DAPI staining. To estimate the

percentage of cell death, CL sections from Stage II (control), 18 h post injection and Stage IV (spontaneous luteolysis) were stained for DAPI. The apoptotic nuclei by DAPI staining were characterized as condensed and small compared to the large nuclei in the normal cells (Fig. 3B). The percentage cell death was higher ($p < 0.05$) than 40% in sections of CL obtained from 18 h post injection and during spontaneous regression (Fig. 3B).

We next examined the biochemical integrity of DNA of CL collected from untreated animals both during Stage II and IV of estrous cycle to determine DNA laddering, considered as hallmark of apoptosis. Agarose gel electrophoresis and ethidium bromide staining of genomic DNA isolated from CL during Stage IV of estrous cycle i.e., during spontaneous regression showed characteristic pattern of DNA laddering indicative of apoptotic cell death in the luteal tissue (Fig. 4A). On the other hand, DNA isolated from CL collected from Stage II of estrous cycle did not show laddering (Fig. 4A). When genomic DNA isolated from CL before and at different time points after $\text{PGF}_{2\alpha}$ analog injection were analyzed for low molecular weight fragments, the results indicated that DNA fragments could be visualized consistently at 18 h post treatment (Fig. 4B). Quantitation of DNA fragments indicated that the low molecular weight DNA labeling increased ($p < 0.05$) >500% at 18 h compared to the low molecular weight DNA labeling observed in CL collected from control animals (Fig. 4C and D). Low molecular weight DNA labeling did not increase ($p > 0.05$) significantly in genomic DNA isolated from buffalo cows at 4 and 12 h post injection (Fig. 4D).

Involvement of MAP kinases during $\text{PGF}_{2\alpha}$ -induced apoptosis

Figure 5 shows representative immunoblot analysis and integrated arbitrary densitometric units data of phospho- and total p38 MAPK levels (expressed as % of 0 h) from CL collected at different time points post PGF_{2α} treatment. Phospho-p38 levels appeared to be higher ($p > 0.05$) at 4 and 12 h after PGF_{2α} analog injection compared to the CL collected from the control animals, and the levels were ~2-4 fold higher ($p < 0.05$) at 18 h. Total p38 levels, although tended to be higher at 18 h, did not increase ($p > 0.05$) significantly at the time points tested (Fig. 5).

Immunoblot analyses of phosphorylation dependent [phospho- (p)] and independent (total) levels of JNK1/2 are presented in Fig. 6 (A & B). Phospho-JNK1 levels increased ($p < 0.05$) 4 fold at 4 and 12 h after PGF_{2α} analog injection compared to the level at 0 h, and this increased ($p < 0.05$) further to ~14 fold at 18 h (Fig. 6A). However, JNK1 levels did not change ($p > 0.05$) significantly from levels found at 0 h (Fig. 6A). Similarly, although the p-JNK2 from CL collected at different time points after tended to be higher ($p < 0.05$), but the levels were not significantly different from that observed in CL of control animals (Fig. 6B). Phosphorylation independent levels were not significantly different ($p < 0.05$) between the control and treated animals (Fig. 6B).

We then examined the immunohistochemical staining for phospho-JNK (p-JNK) using an antibody that recognizes both the p-JNK1 and p-JNK2 in sections of CL from 0 and 18 h after PGF_{2α} analog injection. Typical sections are presented in Fig. 7. Consistent with the increased p-JNK1 and to a lesser extent p-JNK2 levels, nuclear staining of p-JNK increased progressively starting from 4 h and was highest at 18 h, a maximal time point after treatment observed in the present study (Fig. 7). The immunohistochemistry findings confirm the immunoblot analysis data of profound

increase in p-JNK levels during PGF_{2α} induced apoptosis and further suggests that the phospho-JNK levels are localized mainly in the nuclei after PGF_{2α} analog injection.

The total ERK1 & ERK2 (44 kDa and 42 kDa respectively) were evaluated for their involvement during the PGF_{2α}-induced apoptosis and the results are presented in Fig. 8. The total ERK1/2 levels did not vary ($p > 0.05$) after PGF_{2α} analog injection (Fig. 8). When the membranes were probed with phospho-ERK antibodies, the signals appeared very weak (despite using lysate protein samples up to 300 μg) and the signals from CL lysates after treatment appeared lower (data not shown).

DISCUSSION

To date, the nature of luteal regression has not been studied in the buffalo cow. Our findings in the present study demonstrate for the first time in this species, 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 changes such as endocrinological and morphological observations noted in the buffalo cow is quite similar to those made in the cattle [4]. We have recently quantitated PF receptors in the CL of the buffalo cow [31], and the affinity and number of receptors observed are quite similar to that of the pattern reported during different stages of the estrous cycle of the cattle [32]. It is not surprising then exogenous administration of PGF_{2α} also induced luteolysis by the process apoptosis in the mid-estrous cycle of the buffalo cow. The P₄ profile and the time course of appearance of DNA laddering are strikingly similar to the PGF_{2α}-induced luteolysis reported previously for the cattle and sheep [6-8].

In order to determine the role of StAR during $\text{PGF}_{2\alpha}$ -mediated suppression of steroidogenesis, we examined the mRNA expression and protein levels in the CL obtained from control and $\text{PGF}_{2\alpha}$ -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 [32] studied mRNA expression and protein levels in the CL of the cattle and observed a 50% decline in mRNA within 12 h and the levels were undetectable by 24 h after $\text{PGF}_{2\alpha}$ injection. In the ewe, Juengel et al. [34] reported that, even though serum P_4 levels declined within 4 h after $\text{PGF}_{2\alpha}$ injection, but the decline in StAR mRNA levels were apparent only at 12 h. Recently, Tsai et al [35] reported that StAR mRNA in the bovine CL declined as early as 4 h after $\text{PGF}_{2\alpha}$ injection. Of interest is the pattern of decrease in StAR protein observed in the present study that appears to be similar to the time course of decrease in StAR protein levels in the CL during gonadotropin releasing hormone-induced luteolysis of women [36]. Unlike the dramatic fall in the levels of mRNA, the levels of protein declined only to ~50% of control throughout the study period suggest that perhaps there may be additional mechanisms for the continued $\text{PGF}_{2\alpha}$ -induced decrease in P_4 , since a significant level of StAR protein was present despite continued decrease in P_4 concentrations. The time course of decrease in StAR protein after $\text{PGF}_{2\alpha}$ injection noted in the present study is at variance with a study that reported decreased levels within 1 h after $\text{PGF}_{2\alpha}$ injection in the rat CL [37]. Although in the present study, the experiment was not designed to address the site and mechanism of action of $\text{PGF}_{2\alpha}$ on StAR, the dramatic decrease in the transcript appears to suggest that the anti-steroidogenic action of $\text{PGF}_{2\alpha}$ may be mediated through activation of PKC pathway [12-14, 38,39]. It has been

reported that StAR has three phosphorylation sites for PKC and activation of PKC by $\text{PGF}_{2\alpha}$ is expected to decrease and/or block the cholesterol transport and consequently, decreased P_4 biosynthesis [3]. However, as yet there is no experimental evidence to support that view.

The morphological and biochemical data on spontaneous and $\text{PGF}_{2\alpha}$ -induced luteolysis reported in the present study are similar to findings reported by others for the cow [6,7] and sheep [8]. Apoptosis is a multi-step 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. While DNA degradation in apoptotic cell is regarded as part of the executionary phase of apoptosis (i.e., the final events leading to cell death), but the events leading to apoptosis during luteolysis is still not well characterized and is the subject of intense investigation. Moreover, 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]. While the ERK signaling module (activated by mitogenic stimuli) has been shown to play a critical role in cell proliferation, the roles of JNK and p38 MAPK signaling modules (activated by non-mitotic stimuli) are only now beginning to be understood. In the present study, under *in vivo* condition, we demonstrate for the first time activation of JNK and p38 MAPK signaling module in the CL in response to $\text{PGF}_{2\alpha}$ injection. Using *in vitro* cell culture systems involving transformed cell lines, several laboratories have demonstrated that stress stimuli such as UV,

chemicals, shear stress and heat couple these modules to either receptor tyrosine kinases or G proteins [40-43]. Under in vitro conditions, Chen et al. [44] were the first to report stimulation of ERK signaling module by the $\text{PGF}_{2\alpha}$ in bovine luteal cells. While the importance of activation of ERK signaling pathway by $\text{PGF}_{2\alpha}$ or by pharmacological agents that activate PKC in the luteal cells remains to be determined, but the observation of Chen et al. [44] demonstrated the role of MAP kinase cascades in gene transcription in the luteal tissue. Recently, Rueda et al. [45] demonstrated that exposure of bovine luteal cells in vitro to UV irradiation led to activation of phosphorylated forms of JNK, p38 MAPK and attenuation of ERK providing evidence for an important role for intracellular MAP kinase signaling pathways during luteal function and regression. These observations of the present study appear strikingly similar to the findings reported by Rueda et al. [45]. The findings of the present study together with the data of Rueda et al. [45] suggest that luteal cells could be used under in vitro conditions to study the signaling mechanisms involved in the onset of apoptosis, since earlier studies were unable to find correlation between $\text{PGF}_{2\alpha}$ treatment and apoptosis. In the present study, it is not clear whether activated p38 and JNK signaling modules are responsible for the $\text{PGF}_{2\alpha}$ -induced apoptosis, and more studies are required to correlate the two events. However, it should be pointed out that phosphorylated forms of p38 and JNK levels during the first 4-12 h after $\text{PGF}_{2\alpha}$ injection were either similar to control levels or slightly elevated, but with a dramatic increase, especially of p-JNK1, observed at 18 h makes it difficult to say whether these events are causal of apoptosis, since DNA laddering (considered as execution phase of apoptosis) was consistently observed also at 18 h post $\text{PGF}_{2\alpha}$ injection. Nonetheless, the observation that a luteolytic dose of $\text{PGF}_{2\alpha}$ activates JNK and

p38 signaling modules indicates that these kinases may have an important role in the PGF_{2α}-mediated apoptotic cell death in the CL. That the MAP kinases play a critical role in the apoptotic cell death is well established from numerous studies carried out using a variety of cell lines [46-48]. Additionally, the transient transfections studies using activated mutants of MAPKs also confirm important role of JNK pathway in the apoptosis of cells [49].

In conclusion, we demonstrate that the CL of the buffalo cow undergoes regression by the process of apoptosis. Furthermore, the results of this study suggest an important role for MAP kinases in the initiation of onset of PGF_{2α}-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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REFERENCES

1. Niswender GD, Nett TM. Corpus luteum and its control in infraprimate species. In: Knobil E, Neill JD, The Physiology of Reproduction, vol. 1, 2nd ed. New York: Raven Press; 1994:781-816.
2. Zeleznik AJ, Benyo DF. Control of follicular development, corpus luteum function and the recognition of pregnancy in higher primates. In: Knobil E, Neill JD, The Physiology of Reproduction, vol. 2, 2nd ed. New York: Raven Press; 1994:751-782.
3. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* 2000; 80:1-29.
4. Jainudeen MR, Hafez ESE. Cattle and Buffalo. In: Hafez ESE (eds.), *Reproduction in farm animals*, 9th ed. Philadelphia: Lea Febiger Press; 1993:315-329.
5. McCracken JA, Custer EE, Lamsa JC. Luteolysis: a neuroendocrine-mediated event. *Physiol Rev* 1999; 79:263-323.
6. Juengel JL, Garverick HA, Johnson AL, Youngquist RS, Smith MF. Apoptosis during luteal regression in cattle. *Endocrinology* 1993; 132:249-254.
7. Rueda BR, Tilly KI, Botros IW, Jolly PD, Hansen TR, Hoyer PB, Tilly JL. Increased bax and interleukin-1beta-converting enzyme messenger ribonucleic acid levels coincide with apoptosis in the bovine corpus luteum during structural regression. *Biol Reprod* 1997; 56:186-193.
8. Rueda BR, Wegner JA, Marion SL, Wahlen DD, Hoyer PB. Internucleosomal DNA fragmentation in ovine luteal tissue associated with luteolysis: in vivo and in vitro analyses. *Biol Reprod* 1995; 52:305-312.

9. McCormack JT, Friederichs MG, Limback SD, Greenwald GS. Apoptosis during spontaneous luteolysis in the cyclic golden hamster: biochemical and morphological evidence. *Biol Reprod* 1998; 58:255-260.
10. Bacci ML, Barazzoni AM, Forni M, Costerbosa GL. In situ detection of apoptosis in regressing corpus luteum of pregnant sow: evidence of an early presence of DNA fragmentation. *Domest Anim Endocrinol* 1996; 13:361-372.
11. Davis JS, Alila HW, West LA, Corradino RA, Hansel W. Acute effects of prostaglandin F2 alpha on inositol phospholipid hydrolysis in the large and small cells of the bovine corpus luteum. *Mol Cell Endocrinol* 1988; 58:43-50.
12. Wiltbank MC, Guthrie PB, Mattson MP, Kater SB, and Niswender GD. Hormonal regulation of free intracellular calcium concentrations in small and large ovine luteal cells. *Biol Reprod* 1989; 41:771-778.
13. Wiltbank MC, Diskin MG, Flores JA, Niswender GD. Regulation of the corpus luteum by protein kinase C. II. Inhibition of lipoprotein-stimulated steroidogenesis by prostaglandin F2 alpha. *Biol Reprod* 1990; 42:239-245.
14. McGuire WJ, Juengel JL, Niswender GD. Protein kinase C second messenger system mediates the antisteroidogenic effects of prostaglandin F2 alpha in the ovine corpus luteum in vivo. *Biol Reprod* 1994; 51:800-806.
15. Stocco DM. Tracking the role of a star in the sky of the new millennium. *Mol Endocrinol* 2001; 15:1245-1254.
16. Wiltbank MC, Belfiore CJ, Niswender GD. Steroidogenic enzyme activity after acute activation of protein kinase (PK) A and PKC in ovine small and large luteal cells. *Mol Cell Endo* 1993; 97:1-7.

17. Schaeffer HJ, Weber MJ. Mitogen-Activated Protein Kinases: Specific Messages from Ubiquitous Messengers. *Mol Cell Biol* 1999; 19:2435-2444.
18. Allen MP, Linseman DA, Udo H, Xu M, Schaack JB, Varnum B, Kandel ER, Heidenreich KA, Wierman ME. Novel Mechanism for Gonadotropin-Releasing Hormone Neuronal Migration Involving Gas6/Ark Signaling to p38 Mitogen-Activated Protein Kinase. *Mol Cell Biol* 2002; 22:599-613.
19. Smith J, Yu R, Hinkle PM. Activation of MAPK by TRH Requires Clathrin-Dependent Endocytosis and PKC but Not Receptor Interaction with β -Arrestin or Receptor Endocytosis. *Mol Endocrinol* 2001; 15:1539-1548.
20. Burns PD, Mendes JOB Jr, Yemm RS, Clay CM, Nelson SE, Hayes SH, Silvia WJ. Cellular Mechanisms by Which Oxytocin Mediates Ovine Endometrial Prostaglandin $F_{2\alpha}$ Synthesis: Role of G_i Proteins and Mitogen-Activated Protein Kinases. *Biol Reprod* 2001; 65:1150-1155.
21. Ireland JJ, Murphee RL, Coulson PB. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *J Dairy Sci* 1980; 63:155-160.
22. Okuda K, Kito S, Sumi N, Sato K. A study of the central cavity in the bovine corpus luteum. *Vet Rec* 1988; 123:180-183.
23. Hughes FM Jr, Gorospe WC. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology* 1991; 129: 2415-2422.
24. Tilly JL, Hsueh AJ. Microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. *J Cell Physiol* 1993; 154:519-526.

25. Sambrook J, Russell DW. Isolation of high molecular weight DNA from mammalian cells using proteinase K and phenol. In: Molecular cloning, a laboratory manual, vol. 1, 3rd ed. New York: CSHL Press; 2001: 6.4-6.11.
26. Clark BJ, Wells J, King SR, Stocco DM. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *J Biol Chem* 1994; 269:28314-28322.
27. Church GM, Gilbert W. Genomic sequencing. *Proc Natl Acad Sci* 1984; 81:1991-1995.
28. Vara Prasad MVVS, Dermott JM, Heasley LE, Johnson GL, and Dhanasekaran N. Activation of Jun Kinase/Stress-activated Protein Kinase by GTPase-deficient Mutants of G α 12 and G α 13. *J Biol Chem* 1995; 270:18655-18659.
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72:248-254.
30. Selvaraj N, Medhamurthy R, Ramachandra SG, Sairam MR and Moudgal NR. Assessment of Luteal Rescue and Desensitization of Macaque corpus luteum brought about by human chorionic gonadotrophin and deglycosylated human chorionic gonadotrophin treatment. *J Biosci* 1996; 21:497-510.
31. Yadav VK, Natarajan T, Medhamurthy R. Increased stress activated protein kinase activity during PGF 2α -mediated apoptotic cell death in buffalo corpus luteum. In: XXV All India Cell Biology Conference; 2001; Bangalore, India. Abstract 59.
32. Wiltbank MC, Shiao TF, Bergfelt DR, Ginther OJ. Prostaglandin F 2 alpha receptors in the early bovine corpus luteum. *Biol Reprod* 1995; 52:74-78.

33. Pescador N, Soumano K, Stocco DM, Price CA, Murphy BD. Steroidogenic acute regulatory protein in bovine corpora lutea. *Biol Reprod* 1996; 55:485-491.
34. Juengel JL, Haworth JD, Rollyson MK, Silva PJ, Sawyer HR, Niswender GD. Effect of dose of prostaglandin F (2alpha) on steroidogenic components and oligonucleosomes in ovine luteal tissue. *Biol Reprod* 2000; 62:1047-1051.
35. Tsai SJ, Kot K, Ginther OJ, Wiltbank MC. Temporal gene expression in bovine corpora lutea after treatment with PGF₂alpha based on serial biopsies in vivo. *Reproduction* 2001; 121:905-913.
36. Devoto L, Kohen P, Gonzalez RR, Castro O, Retamales I, Vega M, Carvallo P, Christenson LK, Strauss JF 3rd. Expression of steroidogenic acute regulatory protein in the human corpus luteum throughout the luteal phase. *J Clin Endocrinol Metab* 2001; 86:5633-5639.
37. Fiedler EP, Plouffe L Jr, Hales DB, Hales KH, Khan I. Prostaglandin F(2alpha) induces a rapid decline in progesterone production and steroidogenic acute regulatory protein expression in isolated rat corpus luteum without altering messenger ribonucleic acid expression. *Biol Reprod* 1999; 61:643-650.
38. Juengel JL, Meberg BM, Turzillo AM, Nett TM, Niswender GD. Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea. *Endocrinology* 1995; 136:5423-5429.
39. Sandhoff TW, McLean MP. Repression of the rat steroidogenic acute regulatory (StAR) protein gene by PGF₂alpha is modulated by the negative transcription factor DAX-1. *Endocrine* 1999; 10:83-91.

40. Kitagawa D, Tanemura S, Ohata S, Shimizu N, Seo J, Nishitai G, Watanabe T, Nakagawa K, Kishimoto H, Wada T, Tezuka T, Yamamoto T, Nishina H, Katada T. Activation of extracellular signal-regulated kinase by ultraviolet is mediated through Src-dependent epidermal growth factor receptor phosphorylation. Its implication in an anti-apoptotic function. *J Biol Chem* 2002; 277:366-371.
41. Li YS, Shyy JY, Li S, Lee J, Su B, Karin M, Chien S. The Ras-JNK pathway is involved in shear-induced gene expression. *Mol Cell Biol* 1996; 16:5947-5954.
42. Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ. JNK1: a Protein Kinase Stimulated by UV Light and Ha-Ras That Binds and Phosphorylates the c-Jun Activation Domain. *Cell* 1994; 76:1025-1037.
43. Raingeaud J, Gupta S, Dickens M, Han J. Pro-inflammatory Cytokines and Environmental Stress Cause p38 Mitogen-activated Protein Kinase Activation by Dual Phosphorylation on Tyrosine and Threonine. *J Biol Chem* 1995; 270:7420-7426.
44. Chen DB, Westfall SD, Fong HW, Roberson MS, Davis JS. Prostaglandin F₂α stimulates the Raf/MEK1/mitogen-activated protein kinase signaling cascade in bovine luteal cells. *Endocrinology* 1998; 139:3876-3885.
45. Rueda BR, Hendry IR, Ndjountche L, Suter J, Davis JS. Stress-induced mitogen-activated protein kinase signaling in the corpus luteum. *Mol Cell Endocrinol* 2000; 164:59-67.
46. Chen YR, Meyer C F, Tan TH. Persistent Activation of c-Jun N-terminal Kinase 1 (JNK1) in γ Radiation-induced Apoptosis. *J Biol Chem* 1996; 271:631-634.

47. Coso OA, Teramoto H, Simonds WF, Gutkind JS. Signaling from G Protein-coupled Receptors to c-Jun Kinase Involves $\beta\gamma$ Subunits of Heterotrimeric G Proteins Acting on a Ras and Rac1-dependent Pathway. *J Biol Chem* 1996; 271:3963-3966.
48. Murga C, Zohar M, Teramoto H, Gutkind JS. Rac1 and RhoG promote cell survival by the activation of PI3K and Akt, independently of their ability to stimulate JNK and NF-kappaB. *Oncogene* 2002; 21:207-216.
49. Davis RJ. Signal Transduction by the JNK Group of MAP Kinases. *Cell* 2000; 103:239-252.

Legends to Fig 1-7

Figure 1. Serum concentrations of P_4 (Mean \pm SEM) before and after Tiaprostone (synthetic analog of $PGF_{2\alpha}$) injection. Bars with different letters are different ($p < 0.05$) from each other.

Figure 2. A, StAR mRNA expression and ethidium bromide stained 28S and 18S RNA bands and B, StAR 30 kDa protein levels in CL tissues retrieved from buffalo cows before and after $PGF_{2\alpha}$ analog injection. Bars with different letters are different ($p < 0.05$) from each other.

Figure 3. A, Representative H and E-stained section of CL retrieved from control (0 h) and $PGF_{2\alpha}$ analog treated (4, 12 and 18 h) buffalo cows. Note the appearance of morphologically different cells at 18 h post $PGF_{2\alpha}$ treatment. Solid arrowheads indicate nuclei of abnormal cells. B, A DAPI stained CL tissue from control (0 h), 18 h after $PGF_{2\alpha}$ analog injection, and from Stage IV of estrous cycle (spontaneous luteolysis; SL). Open arrowheads indicate apoptotic nuclei. Bars with different letters indicate statistical significance ($p < 0.05$). Magnification, 40X (Fig. 3A) and 100X, zoom 2.6 (Fig. 3B)

Figure 4. Genomic DNA isolated from corpora lutea obtained from control (Stage II of estrous cycle), during spontaneous luteolysis (Stage IV of estrous cycle) and at different time points after $PGF_{2\alpha}$ analog injection, was subjected to either qualitative (A and B) or quantitative analysis (C). Note that agarose gel electrophoresis and ethidium bromide staining of DNA obtained from SL clearly show DNA laddering 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 percent change from the control

(time 0 h). Bars with different letters above them are significant ($p < 0.05$) from each other.

Figure 5. Immunoblot analysis of phospho-p38 (p-p38) and total p38 (p38) before and after $\text{PGF}_{2\alpha}$ treatment. Corpus luteum tissue lysates (100 μg) were resolved on SDS-PAGE, transferred to PVDF membrane and probed with phospho-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 was used per experiment). The arbitrary densitometric units from time 0 h were set as 100% and other time points were expressed in relation to time 0 value.

Figure 6. Immunoblot analysis of phospho-JNK1/2 (p-JNK1/2) and total JNK1/2 (JNK1/2) before and after $\text{PGF}_{2\alpha}$ analog treatment. Corpus luteum tissue lysates (100 μg) were resolved on SDS-PAGE, transferred to PVDF membrane and probed with p-JNK1/2, stripped and reprobed with JNK1/2 antibody. The blots shown are from one of three independent experiments (CL from one animal from each time point was used per experiment). The arbitrary densitometric units from 0 h were set as 100% and the arbitrary units in other time points were expressed in relation to 0 h value.

Figure 7. Representative sections of immunohistochemical staining for phospho-JNK. The corpus luteum was collected at 0, 4, 12 and 18 h post $\text{PGF}_{2\alpha}$ analog injection in the buffalo cows. Phospho-JNK was detected using p-JNK antibody with FITC-conjugated goat anti-rabbit antibody. Magnification 100X.

Figure 8. Immunoblot analysis of total ERK1/2 before and after $\text{PGF}_{2\alpha}$ analog treatment. The blot shown is from one of three independent experiments (CL from one animal from each time point was used per experiment). The arbitrary densitometric units from time 0

h were set as 100% and the arbitrary units in other time points post $\text{PGF}_{2\alpha}$ injection were expressed in relation to time 0 h value.

Fig. 1

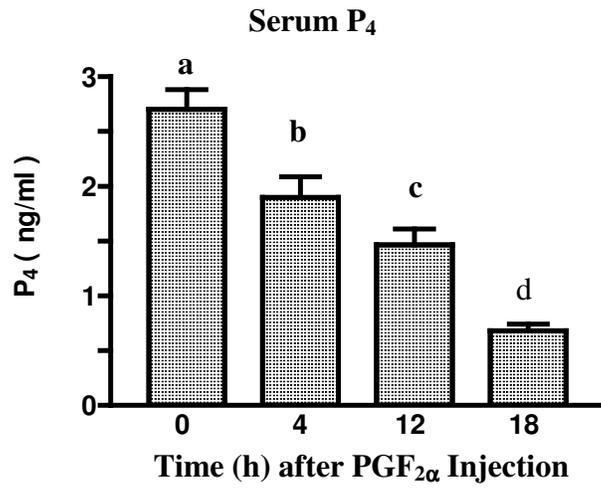


Fig. 2

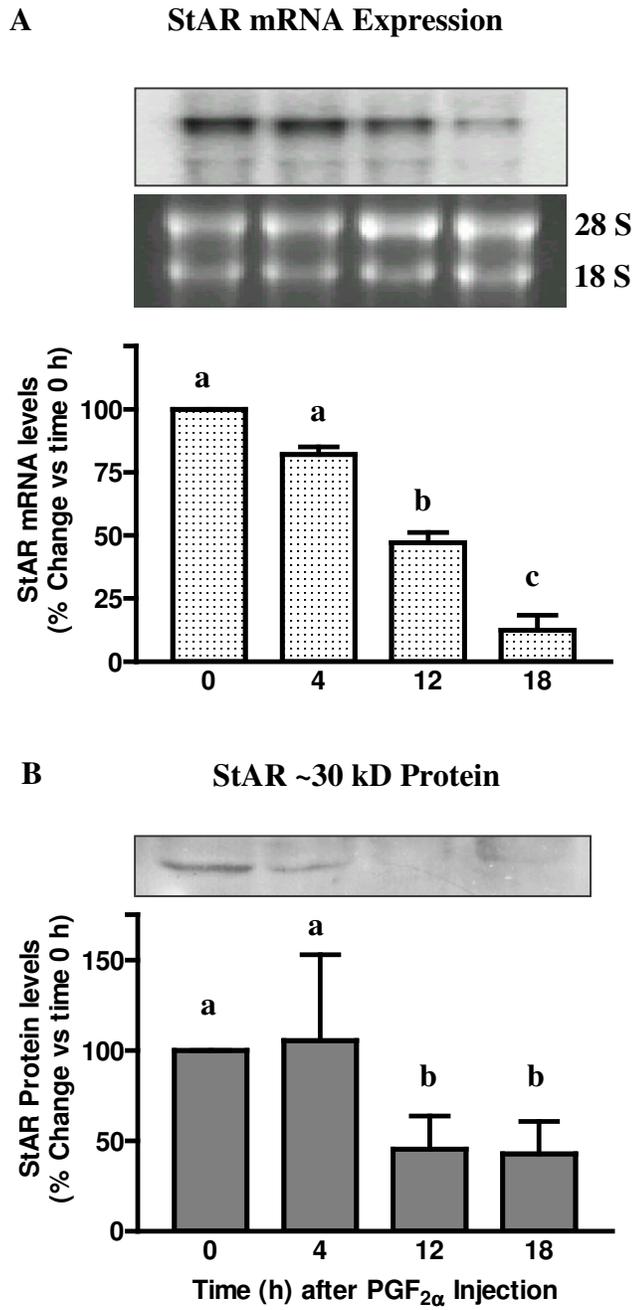


Fig. 3

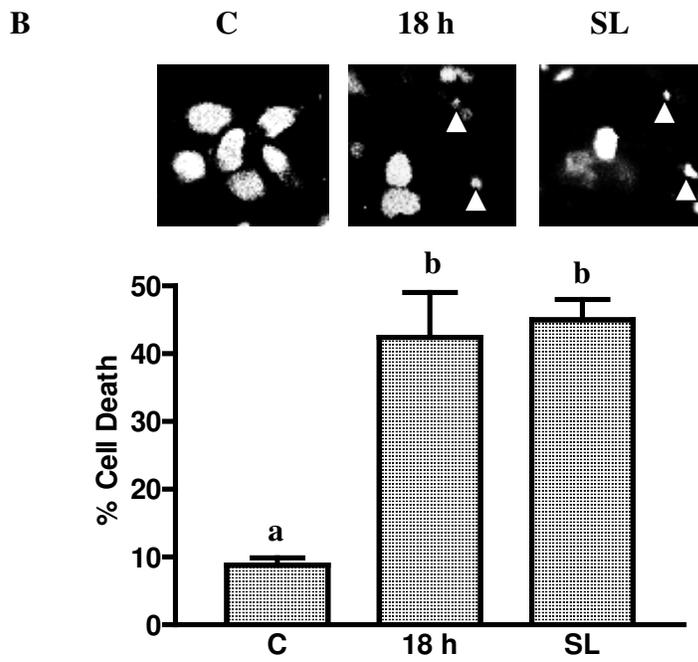
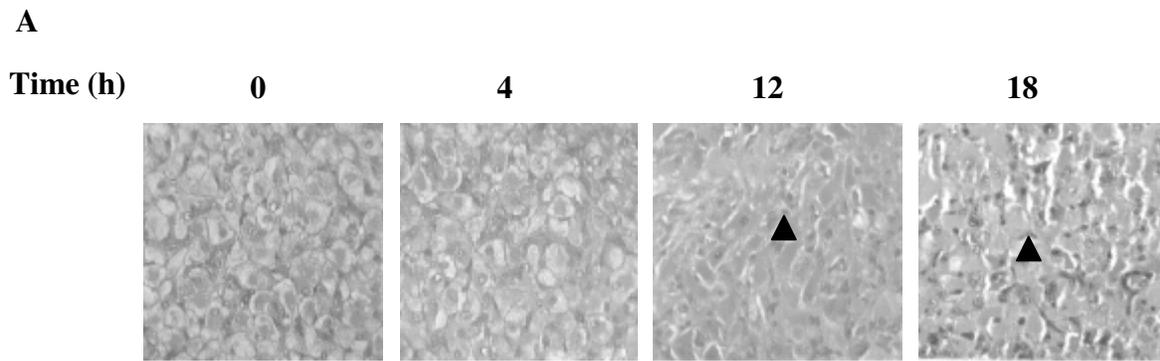


Fig. 5

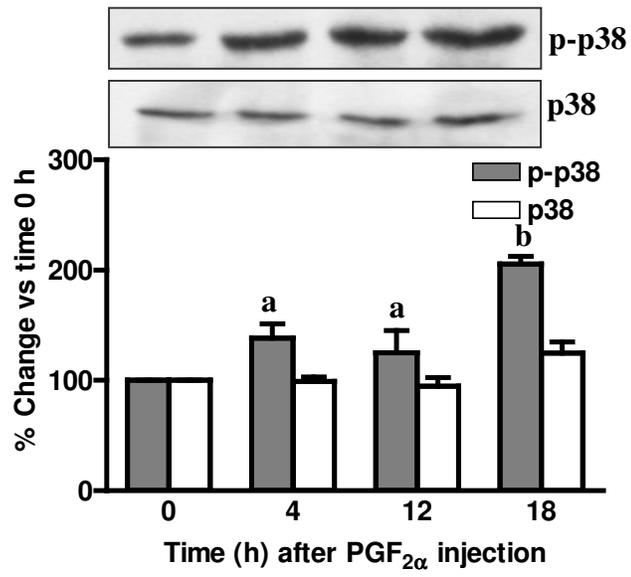


Fig. 6

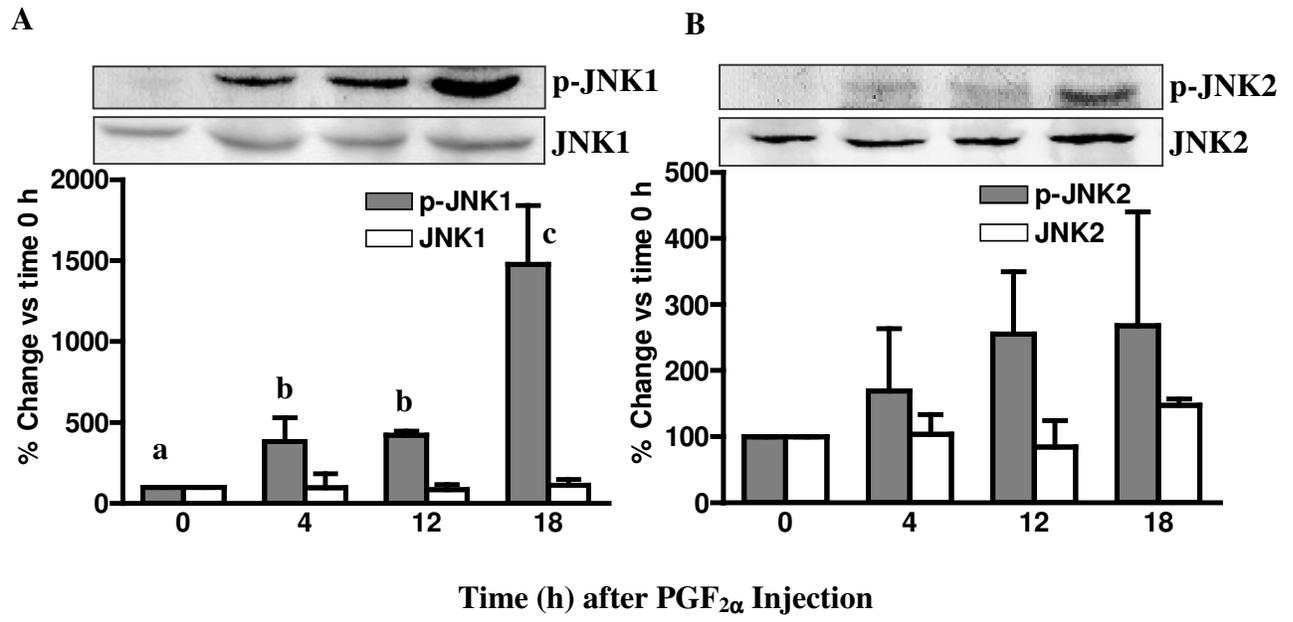


Fig. 7

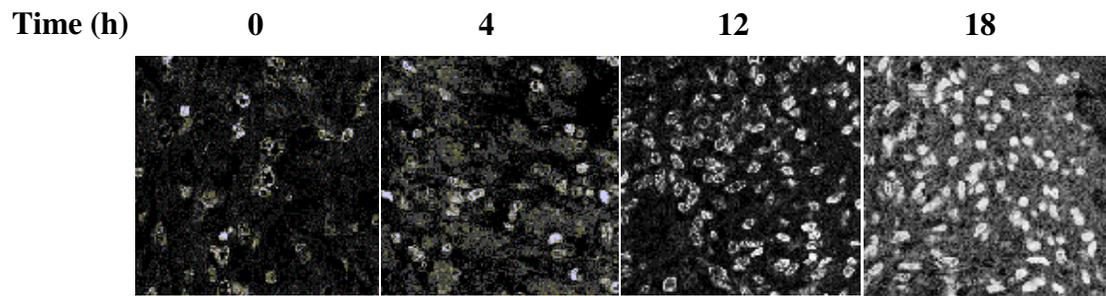


Fig. 8

