Temporal Relationships Between Stress Protein Induction, Progesterone Withdrawal, and Apoptosis in Corpora Lutea of Ewes Treated with Prostaglandin F$_{2\alpha}$

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ABSTRACT: Immunofluorescent staining was used to detect in situ alterations in inducible heat shock (stress) protein (HSP)-70 production and DNA fragmentation (indicative of apoptotic cell death) in luteal tissues obtained from sheep after in vivo administration of a luteolytic dose of PGF$_{2\alpha}$. Accumulation of HSP-70 was localized to PG-sensitive (i.e., large) luteal cells. Cytoplasmic HSP-70 increased within 2 h after treatment. Luteal concentrations of progesterone decreased precipitously thereafter. Expression of HSP-70 diminished by 16 h. An increase in immunostained digoxigenin-labeled DNA was not detected until the onset of structural involution at 16 h; both large and small steroidogenic cells were affected. It is suggested that HSP-70 induction by PGF$_{2\alpha}$ is a prelude to steroid depletion and active apoptotic death of luteal cells.

Key Words: Stress Proteins, Apoptosis, Luteolysis, Sheep

Introduction

Regression of the corpus luteum is a key event dictating ovarian cyclicity. In certain animals, including sheep, the endogenous uterine luteolytic agent is PGF$_{2\alpha}$. In spite of a plethora of research, the cellular and molecular mechanisms involved in PG-mediated luteal regression have not yet been clearly defined (for reviews, see Behrman et al., 1993 and Michael et al., 1994).

Recent preliminary studies have indicated a possible link between stress protein synthesis and physiological cell death (apoptosis) in luteolysis. There was an acute increase in heat shock protein (HSP)-70 accretion within ovine corpora lutea after administration of PGF$_{2\alpha}$ (McPherson et al., 1993). Also, internucleosomal fragmentation of DNA, the biochemical hallmark of apoptosis (Compton, 1992), was noted in regressive corpora lutea of cattle (Juengel et al., 1993) and rabbits (Dharmarajan et al., 1994). Indeed, there is a relationship between stress protein induction and active cell death in hormone-dependent mammary and prostate tumors (Tenniswood et al., 1992).

The objective of the following experiment was to relate concurrent in situ changes in HSP-70 accumulation and apoptotic cell death within corpora lutea collected from sheep at intervals after in vivo injection of a luteolytic dose of PGF$_{2\alpha}$.

Materials and Methods

Mature western-range ewes were observed daily for estrus in the presence of vasectomized rams. The first day of estrus was considered d 0 of the estrous cycle.

An ovary containing a corpus luteum was removed at euthanasia with a barbiturate overdose on d 10 from 9-h controls and ewes treated 2, 4, or 16 h previously with PGF$_{2\alpha}$ (10 mg of dinoprost tromethamine; The Upjohn Co., Kalamazoo, MI). Six ewes were included in each group. Corpora lutea were immediately dissected from extraneous ovarian tissues, weighed, and cut into small segments (≈ 3 × 3 mm) using a single-edged razor blade. One portion of luteal tissue was frozen in liquid nitrogen and later assayed for progesterone (Niswender, 1973; Rahmanian and Murdoch, 1987; intraassay CV = 5%); the remainder of tissue pieces were immersed in chilled 10% neutral buffered formalin.

Fixed tissues (48 h) were washed in two changes of PBS, dehydrated in a graded series of ethanol (50, 70, 95, 95, 100, 100%), cleared (50% absolute ethanol/50% xylene, 2× xylene), and infiltrated with paraffin. Embedded specimens were sectioned using a rotary microtome at 4 to 6 μm, transferred to subbed microscope slides, and deparaffinized.
A mouse monoclonal antibody specific for the inducible form of HSP-70 (SPA-810; StressGen, Victoria, B.C., Canada) was diluted 1:200 in 3% BSA/PBS and applied to sections for 1 h at room temperature. Primary antibody was localized during a 10-min incubation with a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled antimouse immunoglobulin G (Sigma Chemical, St. Louis, MO).

Apoptotic cells were detected with an Oncor (Gaithersburg, MD) S7110 ApopTag kit according to the instructions of the manufacturer. Briefly, exposed 3'-OH ends of genomic DNA fragments were labeled with digoxigenin-11-d uridine triphosphate by terminal deoxynucleotidyl transferase (TdT) catalysis. Incorporated nucleotide heteropolymers were localized with antidigoxigenin Fab-FITC conjugate.

Sections were counterstained lightly in Evans blue or propidium iodide, dehydrated, and coverslipped. Antibody conjugates or TdT were omitted in negative control reactions.

A minimum of 10 randomly selected sections per ovary were processed for each immunostaining procedure. Areas analyzed were selected at random and photographed (×400; Ektachrome 400 HC; 30-s exposure) under an Olympus BH-2 microscope equipped with a reflected light fluorescence attachment. Numbers of reactive luteal cells were counted with reference to total numbers of respective cells represented. Estimates of diameters were made at 16 h on large cells sectioned through a nucleus (apoptosis immunostaining).

Contrasts of mean values due to time of tissue collections were made by one-way analysis of variance and a protected least significant difference test. Differences were considered significant at $P < .05$.

**Results**

Concentrations of progesterone in luteal tissues relative to PGF$_{2\alpha}$ treatment (0 h) were unchanged at 2 h, decreased by 4 h, and decreased further by 16 h (Figure 1). Numbers of large luteal cells that stained positively for the inducible form of HSP-70 increased abruptly at 2 h (i.e., before the onset of functional regression was detected). Proportions of HSP-reactive cells diminished to 0-h control values by 16 h (Figure 1). Small cells were negative for HSP-immunostaining. An increase in apoptotic large and small luteal cells was detected at 16 h, when luteal progesterone concentrations were least (Figure 1). There was a decrease in luteal weights at 16 h (Figure 1). This seemed to be due at least in part to luteal cell shrinkage. Large cells with an apoptotic nucleus were smaller than unstained cells ($22 \pm 4$ vs $33 \pm 3 \mu$m).

Immunostaining for cytoplasmic HSP was diffuse. Discrete nuclear staining of chromatin degradation was typical of apoptotic cells (Figure 2).

**Discussion**

The results of the present experiment indicate that rapid stress protein responses confined to large PG-responsive cells (Alila and Dowd, 1991; Niswender et al., 1994) precede the onset of functional luteolysis in

![Figure 1. Alterations in luteal variables after injection of prostaglandin F$_{2\alpha}$. Means + standard errors are plotted. An asterisk indicates differences ($P < .05$) from 0 h. HSP = stress protein-70; CL = corpus luteum.](image-url)
Figure 2. Representative light photomicrographs of luteal sections immunostained for inducible stress protein (HSP)-70 (upper left = 0 h, upper right = 2 h) or DNA degradation (bottom = 16 h). Notice the difference in degree of specific immunostaining for large cell HSP-70 between 0 and 2 h. Fragmented DNA was detected in nuclei (fluorescence) of large and small cells at the outset of structural regression. L = large cell; S = small cell.

sheep. Whether this represents a cause-and-effect relationship is still unknown. Khanna et al. (1994) found that blockade of gonadotropin or cyclic adenosine monophosphate-stimulated progestogen production by rat luteal cells was related to HSP-70 induction; the antisteroidogenic action was associated with interference of mitochondrial cholesterol translocation. Classically, stress proteins behave as intracellular chaperones and are thereby involved in protein folding and trafficking (Hartl, 1991; Hightower, 1991; Morimoto, 1993; Welch, 1993). It has been suggested that cell-surface autoantigen presentation mediated by escort stress proteins (Kaufmann, 1990; Young, 1990; De Nagel and Pierce, 1991; Hill Gaston, 1991) may act to alert the immune-inflammatory system to impending luteal demise (McPherson et al., 1993).

Apoptosis is a mode of active physiological or programmed cellular death characterized by macromolecular synthesis, oligonucleosomal fragmentation, and cellular shrinkage. Apoptotic bodies are typically resorbed by adjacent epithelial cells or resident macrophages (Ellis et al., 1991; Fesus et al., 1991; Wyllie, 1993; Barr and Tomei, 1994). In contrast, necrosis is a nongenomic process caused by injury. It is exemplified by loss of plasma membrane integrity, cellular swelling and lysis, and significant inflammation. The relative degrees to which apoptosis and necrosis contribute to the overall mechanics of luteal involution remain to be elucidated. Infiltrative leukocytes probably participate in resorption of dead luteal cells (Paavola, 1979; Murdoch, 1987; Standaert et al., 1991; Petrovska et al., 1992; Wang et al., 1992; Hahnke et al., 1994).

Immunostaining of apoptotic cells in situ is a sensitive technique for monitoring biological reactions that might otherwise be undetectable by standard DNA ladder assays. It has been estimated that a rate of tissue regression as rapid as 25% per day can occur as a result of apoptosis in < 3% of cells at any given time (Bursch et al., 1990). The rather marked increase in apoptotic luteal cells is consistent with the fact that structural regression in sheep is nearly complete within 1 to 2 d (Inskeep and Murdoch, 1980). That apoptosis was evident only after the decrease in luteal progesterone concentrations indicates that this phenomenon is not an initiating factor in luteolysis. The results of the present study are therefore in agreement with the findings of Juengel et al. (1993) that DNA fragmentation in bovine luteal tissues (as measured by DNA agarose gel electrophoresis) occurs after the onset of structural regression. It is tempting to speculate that the coincident detection of apoptosis in large and small cells (luteal involution) is causally related to acute steroid withdrawal.

Implications

This contribution provides new information concerning putative interactions between stress protein induction and apoptosis in prostaglandin-induced luteolysis. An understanding of fundamental mechanisms regulating ovarian cyclicity will be of value in developing novel strategies for controlling animal fertility.

Literature Cited


