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Prostaglandin F_{2α}- and FAS-activating antibody-induced regression of the corpus luteum involves *caspase-8* and is defective in *caspase-3* deficient mice

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Abstract

We recently demonstrated that *caspase-3* is important for apoptosis during spontaneous involution of the corpus luteum (CL). These studies tested if prostaglandin F_{2α} (PGF_{2α}) or FAS regulated luteal regression, utilize a *caspase-3* dependent pathway to execute luteal cell apoptosis, and if the two receptors work via independent or potentially shared intracellular signaling components/pathways to activate *caspase-3*. Wild-type (WT) or *caspase-3* deficient female mice, 25–26 days old, were given 10 IU equine chorionic gonadotropin (eCG) intraperitoneally (IP) followed by 10 IU human chorionic gonadotropin (hCG) IP 46 h later to synchronize ovulation. The animals were then injected with IgG (2 micrograms, i.v.), the FAS-activating antibody Jo2 (2 micrograms, i.v.), or PGF_{2α} (10 micrograms, i.p.) at 24 or 48 h post-ovulation. Ovaries from each group were collected 8 h later for assessment of active *caspase-3* enzyme and apoptosis (measured by the TUNEL assay) in the CL. Regardless of genotype or treatment, CL in ovaries collected from mice injected 24 h after ovulation showed no evidence of active *caspase-3* or apoptosis. However, PGF_{2α} or Jo2 at 48 h post-ovulation and collected 8 h later induced *caspase-3* activation in 13.2 ± 1.8% and 13.7 ± 2.2 % of the cells, respectively and resulted in 16.35 ± 0.7% (PGF_{2α}) and 14.3 ± 2.5% TUNEL-positive cells when compared to 1.48 ± 0.8% of cells CL in IgG treated controls. In contrast, CL in ovaries collected from *caspase-3* deficient mice whether treated with PGF_{2α}, Jo2, or control IgG at 48 h post-ovulation showed little evidence of active *caspase-3* or apoptosis. CL of WT mice treated with Jo2 at 48 h post-ovulation had an 8-fold increase in the activity of *caspase-8*, an activator of *caspase-3* that is coupled to the FAS death receptor. Somewhat unexpectedly, however, treatment of WT mice with PGF_{2α} at 48 h post-ovulation resulted in a 22-fold increase in *caspase-8* activity in the CL, despite the fact that the receptor for PGF_{2α} has not been shown to be directly coupled to *caspase-8* recruitment and activation. We hypothesize that PGF_{2α} initiates luteolysis *in vivo*, at least in part, by increasing the bioactivity or bioavailability of cytokines, such as FasL and that multiple endocrine factors work in concert to activate *caspase-3*-driven apoptosis during luteolysis.

Background

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been implicated as a luteolysin in a number of mammalian species [1–3]. However, the exact mechanism(s) by which $PGF_{2\alpha}$ elicits its response in the corpus luteum (CL) remains unclear. Results from previous studies have implicated so-called death receptor-activating cytokines, such as tumor necrosis factor α (TNF α) and Fas ligand (FasL), as being important mediators of $PGF_{2\alpha}$ -initiated luteolysis [4–6]. Unfortunately, since the vast majority of evidence supporting a role for cytokines in luteal regression has been derived from *in vitro* studies of dispersed cells in culture [5,7–14], it is currently unknown if $PGF_{2\alpha}$ modulates death receptor-coupled signaling pathways in the CL *in vivo*.

At least 29 TNF receptor super family members have been identified in the human [15] some of which have been deemed death receptors either by their action or because they contain the highly homologous amino acid sequence corresponding to death domain (DD) [15,16]. The FAS/FasL system was chosen for study herein because FAS is recognized as a death receptor and indirect evidence suggests that it plays a significant role in luteal regression. For example, FAS immunostaining is observed in human granulosa-lutein cells during the early luteal phase, and progressively intensifies during the mid-luteal phase through the late luteal phase [17]. This expression pattern is also observed in the CL of mice [18] and rats [19,20]. In keeping with the proposal that FAS plays a role in luteolysis, *in vitro* studies have shown that FasL or FAS-activating antibodies induce luteal cell death in the human [10], mouse [5,18], rat [19,20] and cow [21]. Moreover, limited *in vivo* work has demonstrated that intravenous or intraperitoneal administration of FAS-activating antibody causes luteolysis in the mouse [18].

Additional support for a functional role of FAS in luteal regression has been derived from experiments with homozygous *lpr* mice, which have non-functional or minimally-functional FAS [22]. The CL of these mice undergo regression, but at irregular intervals [18]. Similarly, homozygous *gld* mice, which lack expression of functional FasL [22], have an irregular luteal phase similar to that observed in *lpr/lpr* mice [18]. Unfortunately, no work was done to characterize potential apoptosis defects in the CL of these mutants, and thus the basis for the irregular cyclicity remains unknown. Collectively these results suggest that FAS-mediated events are critical to timely progression of the normal estrous cycle, and perhaps to luteal regression, in the adult murine ovary.

Thus it is conceivable that some level of cross-talk occurs between $PGF_{2\alpha}$ -initiated events and death receptor function during the tissue involution process of the CL. Mechanistically,

this would likely involve the activation of *caspases*, a family of aspartic acid-specific cysteine proteases that serve as both initiators (*e.g.*, *caspase-8*) and executioners (*e.g.*, *caspase-3*) of apoptosis in vertebrates [23]. Indeed, we have recently shown using a synchronized ovulation mouse model that activation of *caspase-3* is required for structural involution of the CL [24]. The present study was designed to test the hypothesis that $PGF_{2\alpha}$ -induced luteal regression *in vivo* is mediated through indirect mechanisms involving death receptor-coupled *caspase-8* activation followed by a *caspase-3*-dependent pathway of apoptosis.

Materials and Methods

Animals

Wild-type (WT) and *caspase-3*-deficient female mice (congenic C57BL/6) were generated by mating heterozygous male and female mice [25]. Female mice were genotyped by PCR analysis of tail-snip genomic DNA using specific primers [25]. Timed ovulation was induced in all experiments at day 25–26 postpartum by intraperitoneal injection of mice with 10 IU of eCG; (Professional Compounding Centers of America, Houston, TX) followed by 10 IU hCG; (Serono Laboratories, Randolph, MA) 46 h later [24]. This regime induces superovulation at approximately 10 hours post hCG with development of 8 to 10 CL per ovary. Peak progesterone synthesis occurs at approximately 30 hours post ovulation and levels fall back to preovulatory levels by approximately 60 hours (unpublished). All animal protocols were reviewed and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee, and were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals.

In vivo studies of luteal regression

In these experiments, gonadotropin-synchronized mice (wild-type or *caspase-3*-null) were injected at 24 or 48 h post-ovulation with IgG (2 μ g/mouse, i.v.; a non-activating Anti-FAS antibody; Santa Cruz Biotechnology, Santa Cruz, CA), the hamster monoclonal anti-mouse FAS-activating antibody Jo2 (2 μ g/mouse, i.v.; PharMingen, San Diego, CA) or $PGF_{2\alpha}$ (10 μ g/mouse, i.p.; Lutalyse, Pharmacia-Upjohn Co., Kalamazoo, MI). Ovaries and blood were then collected at 8 h post-injection ($n = 3$ mice per genotype per treatment group). In some experiments, ovaries were fixed in 10% neutral-buffered formalin, paraffin-embedded, serially sectioned (6 μ m) and mounted in order on glass microscope slides. Ovarian mid-sagittal sections were used for immunohistochemical analysis of active *caspase-3* (see below) or *in situ* analysis of DNA fragmentation by TUNEL (see below). In other experiments, CL were homogenized and assessed for *caspase-8* activity (see below). Serum was separated from blood samples

and stored at -80 C until analysis for progesterone concentrations (see below).

Immunohistochemistry

Paraffin-embedded sections were analyzed by immunohistochemistry for the presence of cleaved ("active") *caspase-3*, as previously described [9,24,26,27] using a 1:3,000 dilution of a rabbit polyclonal antibody (CM1; IDUN Pharmaceuticals Inc., La Jolla, CA) that preferentially recognizes cleaved *caspase-3* [9,24,26,27]. Sections were counterstained with hematoxylin and analyzed by light microscopy. Cells exhibiting red-brown cytoplasmic staining from the colorimetric reaction were considered positive for active *caspase-3*. Negative controls (reactions lacking primary antibody) yielded no reaction product (data not shown). Similarly, ovarian tissue samples from *caspase-3* deficient mice exhibited minimal or no immunostaining (data not shown; see [24,26]), consistent with the fact that CM1 recognizes principally *caspase-3* in addition to low cross-reactivity with processed ("active") *caspase-7* [27]. Sections of ovaries from WT and *caspase-3* deficient mice were always processed in parallel within the same assay. For quantitative comparisons, the number of *caspase-3* positive cells was determined by counting CM-1 reactive cells in 3 fields of view (X600) per CL in three separate CL from each of 3 different mice per genotype per treatment group without knowledge of treatment.

In addition, samples from the two groups were subjected to immunohistochemical analysis of FAS protein as per the manufacturer's instructions with slight modifications as listed below. Paraffin sections were subjected to microwave on high power for 10 minutes in sodium acetate, pH6, for antigen retrieval. Sections were incubated with a rabbit polyclonal FAS antibody (1:50; SC-1024; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody, peroxidase conjugated goat anti-rabbit IgG was applied at a dilution of 1:200. The sections were then stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) to identify FAS positive cells. Controls included sections treated in the same manner but incubated with normal rabbit serum instead of primary antibody. All immunohistochemical staining for any given antibody was done on all sections at the same time under the same conditions.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)

The occurrence of apoptosis in luteal and ovarian sections was assessed by monitoring the presence of DNA fragmentation *in situ*, as described previously [28]. Slides were analyzed by light microscopy after light counterstaining with hematoxylin. Cells exhibiting dark brown nuclear staining from the colorimetric reaction were considered

positive for DNA fragmentation. Negative controls, lacking the labeling enzyme, yielded no reaction product (data not shown). Sections of ovaries from WT and *caspase-3* deficient mice were processed for TUNEL in parallel within the same assay. The percent of apoptotic cells was determined by counting TUNEL-positive cells out of the total number of cells per three fields of view (X600) in each of three CL from each of 3 different mice per genotype per treatment group.

Caspase-8 activity assay

Ovaries were removed 8 h post-treatment with IgG, Jo2, or PGF_{2α} and CL were dissected out using a dissecting microscope. All CL from an individual animal were pooled and homogenized in lysis buffer from the ApoAlert Caspase-8 Colorimetric Assay Kit (Clontech, Palo Alto, CA). The protein content was determined using the DC protein determination assay (BioRad, Hercules, CA). Samples were diluted to 2 µg protein/ml with dilution buffer supplied in the kit. Fifty µl of each diluted sample (100 ng protein/sample) was then incubated with 200 µM IETD-pNA substrate for 2 h at 37 C in the dark, and the reaction product was determined by reading the absorbance at 405 nm.

Serum hormone analysis

Progesterone levels were measured in serum according to the manufacturer's instructions using a direct solid phase enzyme-immunoassay (DRG Progesterone ELISA kit; ALPCO, Windham, NH), as previously validated in our laboratory [24].

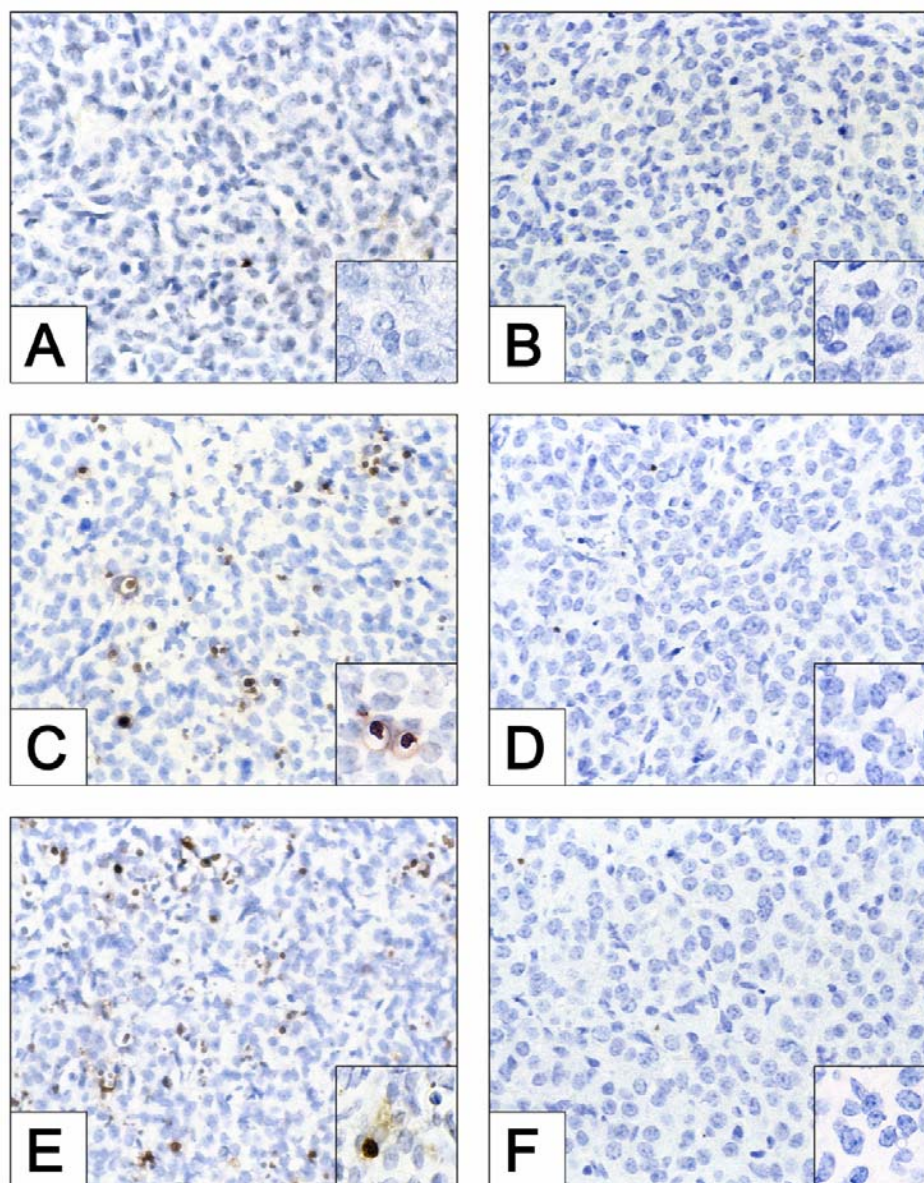
Data Presentation and Statistical Analysis

Each experiment was independently replicated three times with different mice in each experiment. Qualitative data shown are representative of results obtained in the replicate experiments. Quantitative data (mean ± SEM) were analyzed by either Students *t*-test or one-way ANOVA followed by Duncan's New Multiple Range test when differences were observed. A *P* value less than 0.05 was considered statistically significant.

Results

Caspase-3 is required for apoptosis in CL following treatment with Jo2 or PGF_{2α}

There was no evidence of TUNEL-positive cells in the CL of ovaries collected from WT or *caspase-3*-null mice following treatment at 24 h post-ovulation with IgG, FAS-activating antibody (Jo2) or PGF_{2α} (data not shown). The percentage of TUNEL-positive cells in CL of ovaries collected from WT mice following IgG injection at 48 h post-ovulation was 1.48 ± 0.8% (Fig. 1A). By comparison, the CL in ovaries collected from WT mice treated with Jo2 (Fig. 1C) or PGF_{2α} (Fig. 1E) at 48 h post-ovulation exhibited a 10-fold or greater increase in the percentage of TUNEL-positive cells per field of view (Jo2: 14.3 ± 2.5%;

**Figure 1**

Histochemical assessment of apoptosis in CL derived from WT and *caspase-3*-null mice following treatment with IgG, Jo2 or PGF₂α, each administered at 48 h post-ovulation. The ovaries were harvested 8 h post-injection. The data shown depict the incidence of apoptotic (TUNEL-positive, *brown* staining) cells in CL of ovaries derived from WT (A, C, and E) and *caspase-3* deficient mice (B, D, and F) following injection with IgG (A, B), Jo2 (C, D) or PGF₂α (E, F). Original magnifications, × 600. The insets represent a higher magnification (× 1000), demonstrating the presence or absence of TUNEL positive cells. The photomicrographs shown are representative of similar results obtained in at least three independent experiments.

PGF_{2α}: $16.35 \pm 0.7\%$; $P < 0.05$ for both treatments versus IgG controls). In striking contrast to the results with WT mice, there was no significant increase in the percentage of TUNEL-positive cells in CL derived from caspase-3 deficient female mice treated at 48 h post-ovulation with Jo2 (Fig. 1D; 0.32 ± 0.17) or PGF_{2α} (Fig. 1F; 0.22 ± 0.08) when compared with IgG-treated controls (Fig. 1B; $0.06 \pm .003$).

Jo2 or PGF_{2α} increases active caspase-3 enzyme levels in CL of WT mice

There was no evidence of CM1-positive cells in the CL of ovaries collected from WT or caspase-3 deficient mice following treatment at 24 h post-ovulation with IgG, FAS-activating antibody (Jo2) or PGF_{2α} (data not shown). The percentage of CM1-positive cells per field of view in CL of ovaries collected from WT mice following IgG injection at 48 h post-ovulation was low ($1.3 \pm 0.3\%$; Fig. 2A). By comparison, the CL in ovaries collected from WT mice treated with Jo2 (Fig. 2C) or PGF_{2α} (Fig. 2E) at 48 h post-ovulation exhibited a 10-fold or greater increase in the percentage of CM1-positive cells per field of view (Jo2: $13.7 \pm 2.2\%$; PGF_{2α}: $13.2 \pm 1.8\%$; $P < 0.05$ for both treatments versus IgG controls). As anticipated, there was no evidence of CM1-positive cells in CL derived from caspase-3 deficient female mice treated at 48 h post-ovulation with IgG (Fig. 2B), Jo2 (Fig. 2D) or PGF_{2α} (Fig. 2F).

Immunohistochemical detection of FAS in WT and caspase-3 deficient mice

To rule out the possibility that the lack of effect of either Jo2 or PGF_{2α} injected at 24 h post-ovulation was due to the lack of FAS expression in the CL of either WT or caspase-3 deficient mice, ovarian sections from both groups were subjected to immunohistochemical analysis of FAS protein. FAS was evident in luteal tissue of ovaries collected from both WT (Fig. 3B) and caspase-3-null (Fig. 3C) mice at 24 h post-ovulation.

Serum levels of progesterone in caspase-3 deficient versus WT mice

There was no significant difference in the levels of progesterone in WT mice at 8 hours following injection with IgG (4.14 ± 1.09 ng/ml), Jo2 (4.90 ± 2.26 ng/ml), or PGF_{2α} (4.66 ± 1.08 ng/ml) at 24 hours. In addition, no significant difference ($P > 0.05$) in the levels of progesterone was observed in WT versus caspase-3-null (ko) mice at 8 h after injection with IgG (2.76 ± 1.26), Jo2 (wt, 1.25 ± 0.34 ; ko 1.49 ± 0.63) or PGF_{2α} (wt, 2.7 ± 0.8 ; ko, 3.7 ± 0.31) at the 48 h post-ovulation time-points.

Caspase-8 activity

In a final set of experiments, the level of caspase-8 activity was determined in CL isolated from ovaries collected from WT mice 8 h following injection with IgG, Jo2 or

PGF_{2α} at 48 h post-ovulation. Treatment with Jo2 resulted in an 8-fold increase ($P < 0.04$) in caspase-8 activity over the IgG treated controls (Fig. 4). In addition, CL of PGF_{2α}-treated mice had a 22-fold increase ($P < 0.0003$) in caspase-8 activity over controls (Fig. 4).

Discussion

Prostaglandin F_{2α} has long been proposed to disrupt progesterone synthesis, decrease blood flow, increase stress-related signaling, and induce cell death in the CL [2,3]. However, that PGF_{2α} serves as a sole direct regulator of all aspects of luteal regression remains debatable, due in large part to discordant data resulting from species-specific differences in the control of CL function or from studies using *in vivo* versus *in vitro* approaches. For example, administration of PGF_{2α} *in vivo* results in a decline in serum progesterone concentrations and an induction of luteal cell apoptosis in a number of species [29–33]. In contrast, treatment with PGF_{2α} *in vitro* decreases gonadotropin-stimulated progesterone production in cultured rat, ovine, bovine [34–36] and human [37–40] luteal cells, but does not effect cell numbers or viability [35,41]. The loss of pro-apoptotic effects of PGF_{2α} following *ex vivo* removal and chemical dispersal of the CL suggest that either other endocrine factors or cell-to-cell contact is required for PGF_{2α} to kill luteal cells. While interaction between endothelial cells and steroidogenic luteal cells is probably important, recent data support the former possibility as well in that key roles for death receptor ligands, such as FasL and TNFα, in PGF_{2α}-initiated luteolysis have been proposed [5,7,18–20,42]. Careful consideration should also be given to the role of PGF_{2α} as a luteolytic agent in the mouse. Although it is arguably a primary luteolysin in a number of species and may serve as a secondary luteolysin in the rat, its role as a primary or secondary luteolytic agent in the mouse is uncertain. The PGF_{2α} receptor mutant mouse appears to have a normal length estrous cycle, however luteal regression fails to occur during pregnancy [43]. It is not readily clear if the functional and structural components differ in the PGF_{2α} receptor mutant as are described in the caspase-3 mutant mice. Of course it is also possible that there are redundant mechanisms which may serve to propagate the luteolytic signal during the estrous cycle in the absence of a PGF_{2α} receptor.

To more easily study the events underlying the functional and structural components of luteal regression, which are temporally distinct, we have utilized the gonadotropin-synchronized luteal phase mouse model. In this model, we have found that circulating levels of progesterone peak at 30 h post-ovulation and precipitously decline to pre-luteal phase levels by 60 h after ovulation [24]. Structural involution of the CL occurs via caspase-3-mediated apoptosis around 48 h post-ovulation, as evidenced by a dra-

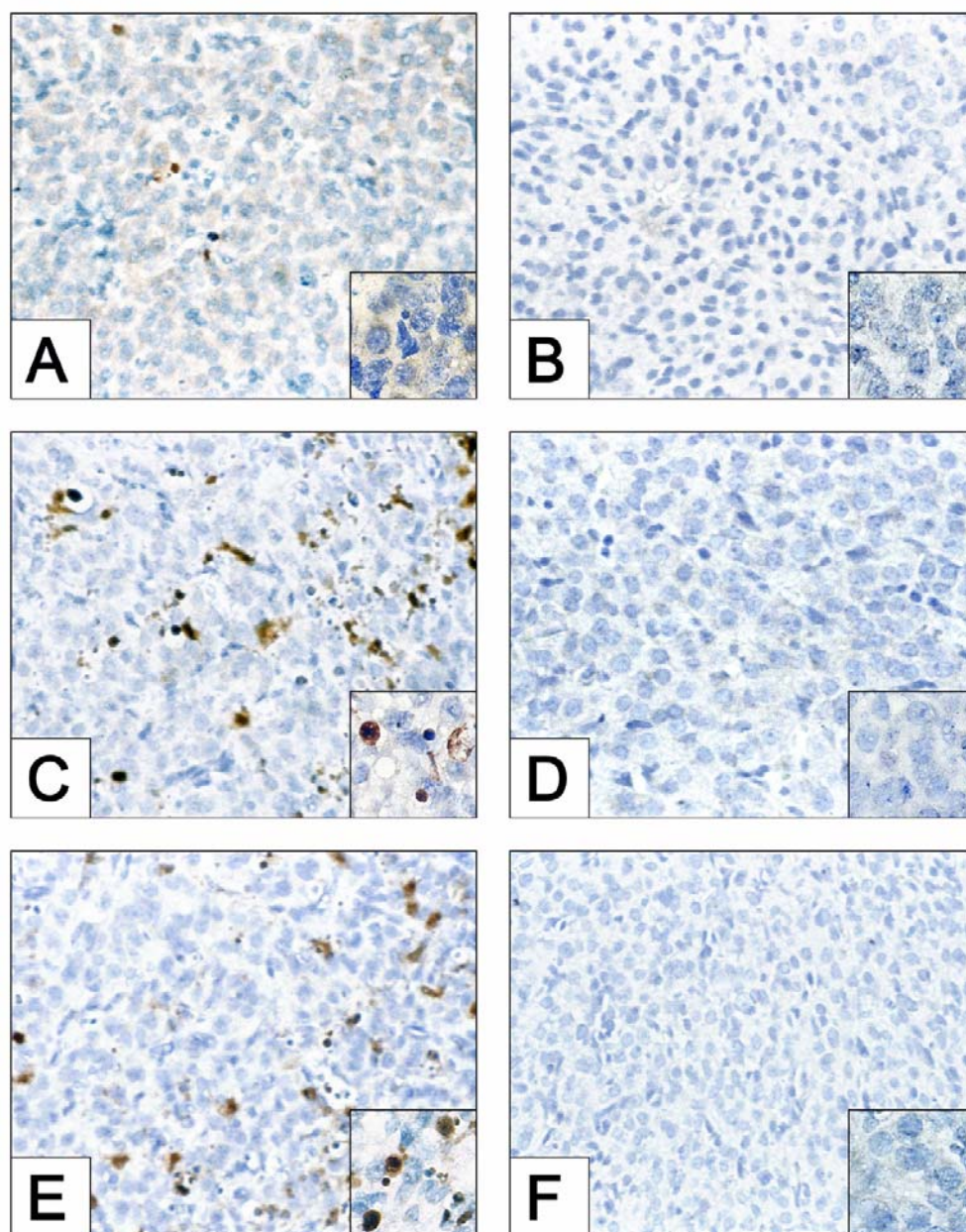
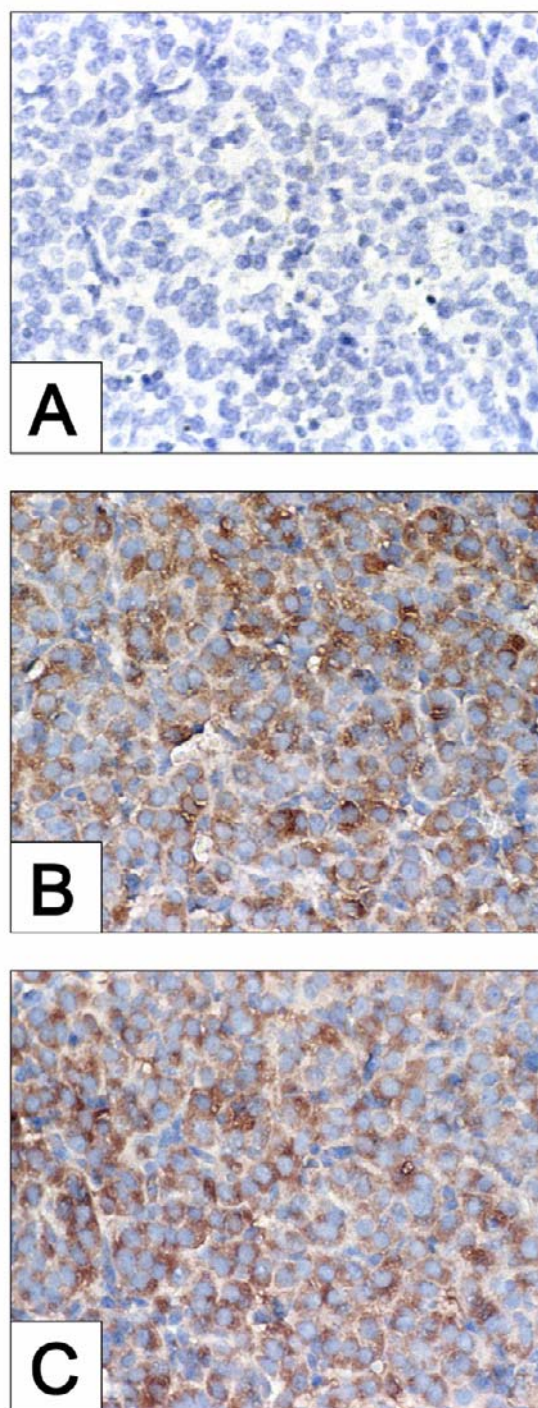


Figure 2

Detection of active *caspase-3* in CL derived from WT and *caspase-3*-deficient mice following treatment with IgG, Jo2 or $\text{PGF}_{2\alpha}$. The presence of active *caspase-3* was evaluated by immunohistochemistry using CM-I antibody in sections of CL derived from WT or *caspase-3*-deficient mice 8 h following injection at 48 h post ovulation with IgG (A and B), Jo2 (C and D) or $\text{PGF}_{2\alpha}$ (E and F). Original magnification: $\times 600$. The insets represent a high magnification (original magnification $\times 1000$) demonstrating the presence or absence of CM-I positive cells (dark brown reaction). Medial sections were obtained from CL from three independent mice for analysis. Photomicrographs shown are representative of identical results in at least three separate experiments.

**Figure 3**

Histochemical assessment of FAS in CL derived from WT and *caspase-3*-deficient mice following treatment with IgG, Jo2 or PGF_{2α} administered 24 h post ovulation and ovaries harvested 8 h post injection. Panel represents a photomicrograph of a CL section derived from WT mouse used as a negative control (no primary antibody). Panel B and C are photomicrographs of CL sections derived from WT and *caspase-3*-deficient mice (respectively) probed with anti-FAS antibody. Original magnification for panels A, B and C × 600.

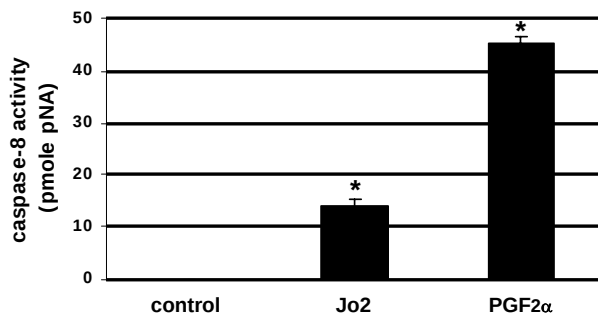


Figure 4

Quantitative analysis of Caspase-8 activity in CL in response to IgG, Jo2 or PGF_{2α} *in vivo*. The level of *caspase-8* activity was determined in CL isolated from ovaries collected from WT mice 8 h following injection IgG (2 µg/IV), Jo2 (2 µg/IV) or PGF_{2α} (10 µg/IP). The asterisk (*) represent differences from the control $P < 0.05$. The experiment was replicated three separate times ($n = 3$) with different groups of mice.

matic increase in TUNEL positive cells concomitant with the rapid decline in progesterone secretion [24]. The requirement for *caspase-3*, determined by the use of gene knockout mice, is consistent with reports that this executioner of apoptosis is expressed in the CL of many species, including the human, bovine, rat and mouse [24,44–46]. Moreover, *caspase-3*-like activity is up-regulated during luteal regression or luteal cell death [9,44,47]. Interestingly, however, whereas the levels of serum progesterone decline in *caspase-3*-null mice similar to their wild-type siblings during luteolysis, structural involution of the CL is suspended for at least 4 d in the mutant animals [24]. This study provided strong evidence for *caspase-3* being paramount to structural involution but not loss of function (decreased progesterone) of the CL.

Using this ovulation-synchronized mouse model, in the present study we found that administration of PGF_{2α} to WT female mice or their mutant siblings (*caspase-3* deficient) at 24 or 48 h post-ovulation had no effect on progesterone levels versus controls. However, administration of PGF_{2α} at 48 h post-ovulation resulted in a dramatic acceleration in the onset of apoptosis when compared to CL in untreated controls. This effect was mirrored by a proportional increase in the incidence of CM1 (active *caspase-3*)-positive cells in the CL, consistent with the hypothesis that this enzyme is important for cell death during structural involution of the CL [24]. These findings were ex-

tended by demonstrating that the CL from *caspase-3* deficient mice were resistant to apoptosis induced by PGF_{2α} exposure *in vivo* when compared to CL of WT type siblings treated in parallel. These data unequivocally demonstrate that *caspase-3* is a required mediator of PGF_{2α}-induced luteolysis.

Of interest, comparable results were obtained following injection of wild-type and *caspase-3* deficient mice at 48 h post-ovulation with Jo2 antibody, an approach used routinely to activate the FAS death receptor *in vivo* [48,49]. The concentration of Jo2 used herein the present study has been shown to be sublethal [48]. The lack of a pro-apoptotic effect of Jo2, or for that matter of PGF_{2α}, when injected at 24 h post-ovulation could not be attributed to lack of FAS since the receptor was present in CL derived from all treatment groups regardless of genotype. Of further interest, both Jo2 and PGF_{2α} induced a significant increase in *caspase-8* activity in CL homogenates. While FAS-mediated signaling clearly involves recruitment and activation of *caspase-8* in all cell types analyzed to date [50], the finding that activity of this enzyme in the CL was so dramatically induced by PGF_{2α} *in vivo* was a surprise. Sequence analysis indicates that the cloned receptor for PGF_{2α} [51,52] does not contain a death-domain (DD), the structural motif found in death receptors that are capable of activating *caspase-8* [50]. Therefore, we can only deduce that the ability of PGF_{2α} to increase *caspase-8* activity is indirectly due to its local modulation of death receptor activation in the CL.

Such a concept would be in keeping with studies of estrous cyclicity in mice lacking functional FAS [18], FasL [18] or TNF receptors [53]. In all three of these mutant mouse lines, luteal phase defects and/or aberrant estrous cycles have been reported. While such findings lend support to the importance of death receptor signaling in normal luteal regression, the disrupted follicular dynamics and ovarian function in these mutants also hindered our attempts to examine *lpr/lpr* mice (Jackson Laboratories) for defects in either PGF_{2α}-induced luteal regression or luteal cell apoptosis. In our hands the *lpr/lpr* mice failed to consistently respond to gonadotropin stimulation as did their wild type siblings (data not shown) lending further support to their irregular cyclicity reported previously [18]. Nonetheless, in light of the data presented, we conclude that PGF_{2α}, along with pro-apoptotic cytokines activated in response to PGF_{2α}, work in concert to orchestrate luteal demise *in vivo* via a *caspase-3*-dependent pathway of apoptosis. Our current efforts are directed at determining the mechanism(s) by which PGF_{2α} up-regulates the activity of death receptor function in the CL, as well as which specific death ligand-receptor family members are expressed, and modulated by PGF_{2α}, in the CL.

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