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Peluffo MC, Stouffer RL, Tesone M. Activity and expression of different members of the caspase family in the rat corpus luteum during pregnancy and postpartum. *Am J Physiol Endocrinol Metab* 293: E1215–E1223, 2007. First published August 28, 2007; doi:10.1152/ajpendo.00261.2007.—Studies were designed to examine the expression and activity of four caspases that contribute to the initial (caspases-2, -8, and -9) and final (caspase-3) events in apoptosis in the rat corpus luteum (CL) during pregnancy (*days 7*, *17*, *19*, and *21* of gestation), postpartum (*days 1* and *4*), and after injection (0, 8, 16, 24, and 36 h) of the physiological luteolysin PGF2α. In addition, the temporal relationship of caspase expression/activity relative to steroid production and luteal regression was evaluated. During pregnancy, the activity of all four caspases was significantly greater on *day 19*, before a decline in CL progesterone (P) and CYP11A1 levels at *day 21* of gestation. The levels of the caspase-3 active fragment (p17, measured by Western blot) also increased at *days 19* and *21* of pregnancy. Immunohistochemical analyses detected specific staining for the caspases in luteal cells (large and small) as well as in endothelial cells. However, the percentage of apoptotic cells did not increase in the CL until postpartum. Following PGF2α injection, there was a significant decrease in CL P by 24 h, although the activity of all four caspases did not increase until 36 h posttreatment. The active p17 fragment of caspase-3 also significantly increased at 36 h post-PGF2α. These results suggest that an increase in the activity of caspases-2, -8, -9, and -3 is associated with the early events of natural luteolysis at the end of pregnancy. Also, the exogenous administration of the luteolysin PGF2α may regulate members of the caspase family.

luteolysis; apoptosis

THE GLAND FORMED FROM the remaining wall of the ovarian follicle after ovulation is called the corpus luteum (CL). The corpus luteum secretes mainly the steroid hormone progesterone (P), which is essential for implantation of the blastocyst and maintenance of pregnancy (9, 59, 61). In the absence of pregnancy in all mammals or at the end of pregnancy in some species, e.g., rodents, rabbits, and goats, the CL ceases to produce P and regresses by luteolysis. Luteolysis can be divided into functional luteolysis, which consists of the loss of its ability to produce P, and structural luteolysis, or morphological regression of the CL. In rodents and domestic animals, prostaglandin F2α (PGF2α) plays a key role in the initiation of functional and probably structural luteolysis (49–51). Luteal regression is associated with programmed cell death, i.e., apoptosis, in many species, including rodents and domestic animals (5, 8, 20, 23, 27, 29, 65, 73), but a causal role has not been established.

Apoptosis occurs mainly through two distinct signaling cascades, the intrinsic and extrinsic pathways. Members of the caspase family are cysteine aspartic acid-specific proteases that appear to be involved in both of these signaling cascades, and their activation is a critical event in apoptosis (3, 28, 33, 40). These proteases are divided into two main categories, the initiators and the effectors (23, 29). Members of the caspase family have been detected in the ovary of different species (7, 16, 17, 34, 53, 54, 69, 72). In addition, data from a caspase-3-deficient mouse model suggest that this effector caspase is pivotal for the timely regression of the CL (11). In ovine studies, the accumulation of caspase-3 mRNA and induction of its activity were observed in the CL following PGF2α treatment (53). Recent evidence from a pseudopregnant rat model shows the importance of caspase-8 and -3 activation in luteal apoptosis during PGF2α treatment (73). However, there are few studies to date on the expression, activity, or regulation of different members of the caspase family in the CL during physiological situations (47, 48).

We hypothesized that, if caspases play a key role in physiological luteolysis (functional and/or structural) in rodents, then caspase protein expression and/or enzyme activity would increase in the CL during late pregnancy or early in the postpartum period, and PGF2α treatment would increase their activities. Therefore, studies were designed to analyze the expression and activity of four caspases believed to contribute to the initial (e.g., caspases-2, -8, and -9) and final (e.g., caspase-3) apoptotic events in the CL during pregnancy and after injection of the physiological luteolysin PGF2α in rats. In addition, the temporal relationship of caspase expression/activity relative to steroid production and luteal regression was evaluated. The different caspases were selected on the basis of their function: one initiator (caspase-9) involved in the intrinsic mitochondrial pathway, one initiator (caspase-8) involved in the extrinsic death receptor pathway, and one initiator (caspase-2) related with both pathways plus the main downstream effector (caspase-3).

MATERIALS AND METHODS

Animals and protocols. Adult female Sprague-Dawley rats received food and water ad libitum and were housed at room temperature (range of 21–23°C) with a 12:12-h light-dark cycle. Animals were killed by CO_2 asphyxiation. The ovaries were removed and cleaned before subsequent assays. All experimental protocols were performed in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and were approved by the Oregon Health and Science University Institutional Animal Care and

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Use Committee and the IBYME ethical committees. Estrous cycling stages were determined by daily examination of vaginal cytology, and rats were mated overnight at proestrus.

Day 1 of gestation was designated as the morning on which spermatozoa were found in a vaginal smear. Rats from specific stages of pregnancy (*days 7*, *17*, *19*, and *21* of gestation) and postpartum (*days 1* and *4*) were killed, and ovaries were collected. In our laboratory, litters were born on day 23 of gestation. Pups were removed after parturition to prevent the postpartum peaks of prolactin (PRL) in mothers. In nonlactating rats, cyclic ovulation occurs every 4 days beginning at parturition, and as a consequence, new CLs are formed. Accordingly, by *days 1* and *4* postpartum, rats displayed two different types of CL in the ovary: the CL of pregnancy and the newly formed CL. These two different types of CLs were easily recognized under a stereoscopic microscope. The CL of pregnancy was larger and appeared whitish compared with the CL from the cycle. Moreover, the CL of pregnancy had thinner veins. Only the CLs of pregnancy were utilized in this study.

An additional group of rats was injected on *day 14* of pregnancy with one dose of PGF2α (400 μg/rat; Biogenesis, Buenos Aires, Argentina), and ovaries were collected at specific times (0, 8, 16, 24, and 36 h) after PGF2α administration. *Day 14* was selected because P production by the rat CL increases during the first 16 days and then declines to very low levels around *days 20–22* of pregnancy, followed by structural luteolysis after parturition (24, 27). Therefore, the pharmacological luteolysis induced by the PGF2α should be clearly separated from the timing associated with the natural luteolysis at parturition.

The CLs were dissected from ovaries under a stereoscopic microscope as previously described in our laboratory (4). Pools of CL from one ovary from each rat (n = 3–4 for each day or time group) were frozen in liquid nitrogen and stored at −80°C for protein isolation. Contralateral ovaries (n = 3–4/group) were fixed in Formalin for analysis of caspases-2, -3, -8, and -9 as well as apoptosis.

Luteal P levels. Steroids were extracted as previously described by our laboratory (31, 56). P content in CL was measured in rats from each group (*days 7*, *17*, *19*, and *21* of gestation and *days 1* and *4* postpartum; n = 3–4 rats/group). A pool of three to four CLs was used to represent luteal P content in each rat. CLs were homogenized in acetone with Ultra-Turrax (IKA Werk, Breisgau, Germany). Also, the P content of the CL from different times (0, 8, 16, 24, and 36 h) after PGF2α injection on *day 14* of pregnancy was measured (n = 3–4). Known quantities of labeled P ([^{3}H]progesterone) in acetone (~3,000 cpm) were added to each sample as internal standard. An aliquot was taken from each homogenate for protein measurement. After homogenization, the samples were centrifuged (1,600 *g* for 10 min), and the resultant supernatant was evaporated to dryness. Following the addition of distilled water and vortexing, the samples were twice extracted with diethyl ether, and the upper ether phase was transferred to conical tubes and again evaporated to dryness. The remaining residue was dissolved in methanol, and, after addition of distilled water, the samples were submitted to a solvent partition with *n*-hexane and dichloromethane; the upper layer was discarded and the lower phase evaporated. Finally, samples were stored in distilled water for later analysis by radioimmunoassay (31).

Caspases-2, -3, -8, and -9: immunohistochemistry. Ovaries were fixed in 10% neutral buffered Formalin for 1 wk. Then, tissues were dehydrated in a series of ethanol solutions (50, 70, and 100%) and paraffin embedded. Four-micrometer sections were deparaffinized and hydrated through xylene and a graded series of ethanol. Afterward, immunohistochemistry (IHC) was performed following the procedure that we previously described (47). Concentrations for caspase-2 (sc-626; Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3 (sc-7148, Santa Cruz Biotechnology), caspase-8 (3020-100, Biovision Research Products), caspase-9 (sc-7885, Santa Cruz Biotechnology) rabbit polyclonal antibodies were 1:500 (caspase-2), 1:1,000 (caspase-3), and 1:200 (caspases-8 and -9). According to the manufacturer, these antibodies recognize both the inactive and the active form of the caspase. For each caspase examined, negative controls lacking primary antibody were processed on adjacent tissue sections.

Western blotting. After 5 min of boiling, extracted proteins from the different CL groups (40 μg protein) were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane using an electroblotting apparatus as previously described (31, 47). The first antibodies were rabbit polyclonal antisera to CYP11A1 (provided by Dr. Anita H. Payne, Stanford University Medical Center, Stanford, CA) and caspase-3 (sc-7148, Santa Cruz Biotechnology) in a concentration of 1:2,000 and 1:200, respectively. Protein expression was quantified by densitometric analysis using Scion Image software for Windows (Scion). Consistency of protein loading was evaluated by staining the membranes with Ponceau S, and the density in each specific band was normalized to the density of an internal control (β-actin, mouse monoclonal, ab6276; Abcam, Cambridge, UK).

Caspases-2, -3, -8, and -9: activity assays. From each ovary, a pool of CL was used to evaluate enzyme activity (n = 3–4 ovaries/time point). A fluorometric assay kit (no. 630225, Clontech) containing fluorogenic substrates specific for different caspases (-2, -3, -8, and -9) immobilized in separate wells was used following the procedure previously described by our laboratories (47, 48). Twenty micrograms (caspase-2 and -3) or 40 micrograms (caspase-8 and -9) of the extracted protein from each pool of CL were added in homogenization buffer to the wells. The plate was incubated in the fluorescence plate reader at 37°C for 3 h, and fluorescence was read every 10 min. The activity was determined by fluorometric detection (excitation, 380 nm; emission, 460 nm), and the negative control (blank, without sample) was subtracted from all of the samples. The 2-h time point was selected for analyses because it was located on the linear section of the curve. Baseline values of negative controls and samples with specific inhibitors did not increase during the 2-h interval.

Apoptotic cell analysis (terminal dUTP nick-end labeling). Serial 4-μm sections of paraffin-embedded fixed ovaries (n = 3–4) from the different days of pregnancy and postpartum were used. Nuclear DNA fragmentation in luteal cells was detected using the DeadEnd Colorimetric TUNEL System (G7130; Promega, Madison, WI), following the manufacturer's instructions with minor modifications, as previously described (47, 48). A microscope with a ×100 objective was used, and three randomly selected fields were analyzed from each CL section (3 CLs per section per ovary, 3–4 ovaries per stage). Results were expressed as percentage of apoptotic cells.

Statistical analysis. Statistical analyses were performed using the ANOVA test for comparison among time points followed by the Student-Newman-Keuls method using the Sigma Stat software package (SPSS, Chicago, IL). Differences were considered significant at $P < 0.05$. Percentage data were analyzed using the ANOVA test (nonparametric, Kruskal-Wallis statistic) for comparison among time points followed by Dunnett's multiple comparison method.

RESULTS

Luteal cell death during pregnancy. First, we evaluated cell death in the CL on *days 7*, *17*, *19* and *21* of pregnancy and on *days 1* and *4* postpartum by using the terminal dUTP nick-end labeling (TUNEL) method. Luteal function on these days was also investigated by measuring intraluteal P levels and the expression of the CYP11A1 enzyme. Nuclear DNA fragmentation in the rat CL was observed in few cells during pregnancy (*days 7–21*) but appeared more prevalent during the postpartum interval (Fig. 1*A*). Cells exhibiting TUNEL staining were expressed as the percentage of apoptotic cells in each group. There was a significant increase ($P < 0.05$) in the percentage of apoptotic cells in the rat CL at *days 1* and *4* postpartum compared with *day 7* (Fig. 1*B*). In addition, the percentage of apoptotic cells was higher at *day 4* postpartum compared with

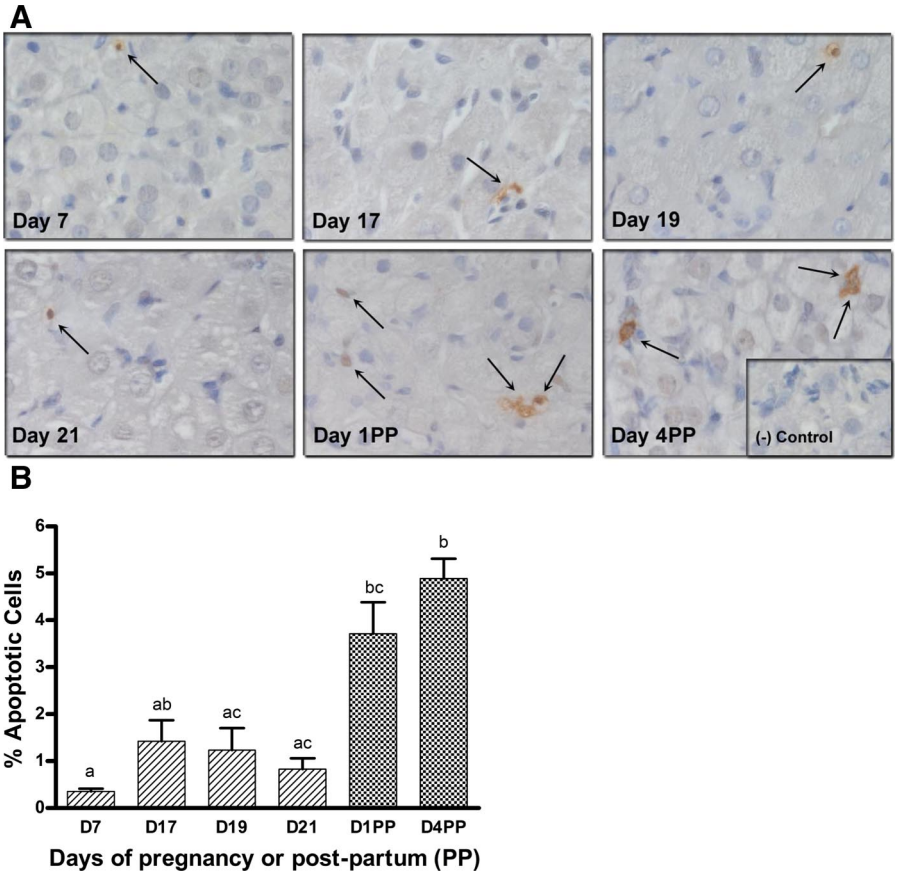

Fig. 1. *A*: sections of the rat corpus luteum (CL) at different time points in pregnancy (*days 7*, *17*, *19*, and *21* of gestation) and postpartum (PP; *days 1* and *4*), with some cells showing advanced signs of apoptosis as positive staining in the terminal dUTP nick-end labeling (TUNEL) assay (arrows). *B*: TUNEL analysis, expressed as the percentage of apoptotic cells in the CL, as pregnancy progressed to PP. Different letters (a, b, c) represent significant differences ($P < 0.05$) between the different days (D) of pregnancy and PP (3 CLs per section per ovary, 3–4 rats per stage).

days 19 and *21* of gestation (Fig. 1*B*). These results indicate that apoptosis in the CL of pregnant rats increases dramatically only after parturition. In contrast, a significant drop in luteal P content was observed by *day 21* of pregnancy (Fig. 2*A*; *day 7*, 157.8 ± 10.7, vs. *day 21*, 49.1 ± 6.7 pg P/μg protein; $P < 0.01$).

Western blotting for CYP11A1 revealed a single band of the expected size (53 kDa) in all samples (Fig. 2*B*). When normalized to β-actin (ACTB, 42 kDa), the highest expression was found at *day 7* of pregnancy, followed by a significant decrease through *days 17*, *19*, and *21* of pregnancy and *day 1* postpartum ($P < 0.01$). Unexpectedly, at *day 4* postpartum, CYP11A1 expression significantly increased compared with *day 1* postpartum and *day 21* of pregnancy ($P < 0.05$).

IHC analysis of caspases-2, -3, -8, and -9 in the rat CL during pregnancy. The expression and cell distribution of caspases-2, -3, -8, and -9 were investigated in paraffin sections of rat ovaries obtained on *days 7*, *17*, *19*, and *21* of pregnancy and on *days 1* and *4* postpartum.

Immunolabeling for initiator caspases-2, -8, and -9 and the main effector, caspase-3, was observed in the CL of pregnancy (Fig. 3). Specific staining for caspase-2 was detected in the cytoplasm and nucleus of small (SL) and large (LL) luteal cells as well as in the endothelial cells but not in the surrounding stroma. IHC for caspase-2 in the CL throughout pregnancy (representative IHC from *days 7* and *19*; Fig. 3) showed a moderate expression followed by a slight decrease at *day 1* postpartum and an increase at *day 4* postpartum. Caspase-8 and -9 expression was also observed in the cytoplasm and in some nuclei of luteal cells (SL and LL), but no staining was found in the endothelial or the stromal cells (Fig. 3). No major changes in protein expression occurred for either caspase-8 or -9 throughout pregnancy. However, their expression decreased at *day 1* postpartum and increased at *day 4* postpartum.

Caspase-3 immunolabeling (Fig. 3) was detected in luteal cells (SL and LL) and endothelial cells, but no staining was found in the stroma. The staining was notably more intense in the nucleus and cytoplasm of the endothelial and SL cells. Higher expression of caspase-3 was observed at *days 7* and *19* of pregnancy with lesser staining by *day 21* of pregnancy (not shown) and *day 1* postpartum, followed by increased staining at *day 4* postpartum. Negative controls performed by omitting the primary antibody showed no staining (*day 4* postpartum; Fig. 3, *inset*).

Caspase-3 proteins in CL during pregnancy and postpartum. Western blot analysis of CL proteins using an anti-caspase-3-specific antibody detected different bands corresponding to procaspase-3 (36 kDa) and different cleavage products, notably the p20 fragment (20 kDa) and the active fragment p17 (17 kDa) (see Fig. 4*B*). Cleavage of this protease occurred in the rat CL throughout pregnancy (*days 7*, *17*, *19*, and *21*) and postpartum (*days 1* and *4*). There were no significant differences in the relative procaspase-3 levels during pregnancy or postpartum. However, levels of the active fragment (p17), expressed as a ratio to procaspase-3, significantly increased in the CL at *days 19* and *21* of pregnancy ($P < 0.05$) (Fig. 4*A*), suggesting that an increase in caspase-3 activity could play an important role in the initiation of luteolysis toward the end of pregnancy.

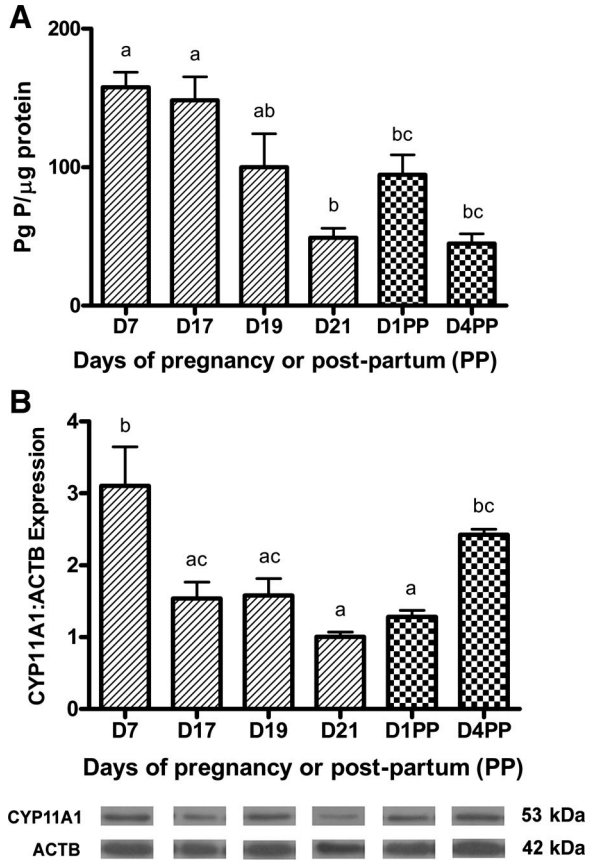

Fig. 2. *A*: progesterone (P) levels (means ± SE, pg P/μg protein) in the CL during pregnancy (*days 7*, *17*, *19*, and *21* of gestation) and PP (*days 1* and *4*). Different letters over SE bars indicate significant differences ($P < 0.05$) between groups ($n = 3$–4/group). *B*: Western blots of CYP11A1 in the rat CL throughout pregnancy or PP. Different letters (a, b, c) represent a significant difference ($P < 0.05$) in CYP11A1 protein levels (means ± SE) between groups ($n = 3$–4/group). *Bottom*: gels with bands corresponding to CYP11A1 (53 kDa) and the internal control, β-actin (ACTB, 42 kDa).

Caspase (caspases-2, -8, -9, and -3) activity in the CL during pregnancy and postpartum. To examine whether the activity of caspases-2, -8, -9, and -3 correlated with the changes in luteal function, we determined their activity in the CL throughout pregnancy. The activity for the initiator caspases (caspases-2, -8, and -9) as well as the effector caspase (caspase-3) showed a similar pattern during pregnancy and postpartum (Fig. 5), peaking at *day 19* and/or *21* of gestation ($P < 0.05$, compared with *day 7*) and then declining by *day 1* postpartum.

Effect of PGF2α on caspase (caspases-2, -8, -9, and -3) activity and P content in CL. Our results suggest that an increase in the activity of caspases-2, -8, -9, and 3 is associated with the initiation of functional luteolysis (decrease in luteal P content) near the end of pregnancy. Administration of PGF2α to pregnant rats also rapidly decreases P levels in the rats (62). Therefore, we examined whether PGF2α treatment affects caspase activity when administered to *day 14* pregnant rats. Following PGF2α administration (Fig. 6), the activity of all four caspases tended to decline for 8–24 h, but this was only significant for caspase-3 at 16 h post-PGF2α. By 36 h after PGF2α administration, the activity of the four caspases significantly increased ($P < 0.05$). Moreover, relative procaspase-3 protein levels decreased significantly by 36 h after PGF2α administration ($P < 0.05$, compared with 0 h). Nevertheless, a significant increase in the ratio of the active fragment p17 to the procaspase was observed in the 36-h group ($P < 0.05$; Fig. 7).

P content was measured in isolated CL at different times (0, 8, 16, 24, and 36 h) after PGF2α administration (Fig. 8). A significant decrease (0 h, 112.8 ± 18.8, vs. 24 h, 30.1 ± 5.1 pg P/μg protein; $P < 0.01$) was observed by 24 h postinjection, with an apparent rebound at 36 h.

DISCUSSION

This study provides the first comprehensive examination of protein expression and activity of multiple caspases in the rat CL during pregnancy and postpartum, as well as the temporal relationship with caspase expression and/or activity relative to steroid production and luteal regression. In addition, it demonstrates the effect of exogenous PGF2α on the activation of different initiators and the main effector caspase in the CL of pregnant rats.

Protein expression of the four caspases selected for study was observed in the CL throughout pregnancy and postpartum. But individual caspases exhibited different patterns of expression and cell localization during pregnancy and postpartum. We recently reported the expression and activity of different caspases in the CL during the natural menstrual and estrous cycle of the monkey and rat, respectively (47, 48). Interestingly, caspase-2 and -3 expression was observed in the cytoplasm and nucleus of SL and LL as well as in the endothelial cells, whereas caspases-8 and -9 were only expressed in luteal cells (SL and LL). The fact that endothelial cells express only the initiator caspase-2 (but not caspase-8 or -9) suggests a specific involvement of this initiator caspase in this cell type, which may have a different regulatory mechanism of apoptosis. Recent studies demonstrated that endothelial cells undergo apoptosis and suggested its activation through the TNF-α pathway (19, 52). However, this protease is not only related to the death receptor pathway (2, 18) but also to the mitochondria pathway (27, 28). In addition, caspase-2 may act as both a positive and a negative cell death effector, depending on cell lineage and stage of development (6).

In contrast, we found a similar pattern in the enzyme activity of the four caspases during pregnancy and postpartum. Maximal activity was observed between *days 19* and *21* of pregnancy. Takiguchi et al. (64) demonstrated a similar increase in caspase-3 activity on *day 21* of pregnancy. The increases in caspase (caspases-2, -3, -8, and -9) activity and levels of the active p17 fragment of caspase-3 were before or coincident with the decrease in luteal CYP11A1 and progesterone content. These results suggest that early caspase activation precedes and may induce the drop of progesterone. Similar results were obtained with CL during the natural menstrual cycle of the rhesus monkey (48), where the highest levels of caspase activities were observed at the mid-late luteal phase. At this stage, the monkey CL is on the verge or just beginning to regress, and progesterone levels are still high. However, these results are not in agreement with reports proposing that progesterone suppresses the action of the main effector, caspase-3 (42, 63), and this idea cannot be discarded.

The activation of caspases delineates the "point of no return" in the apoptotic pathway (13, 68). However, these proteases may have other "nonapoptotic" actions in cells. Caspase-6

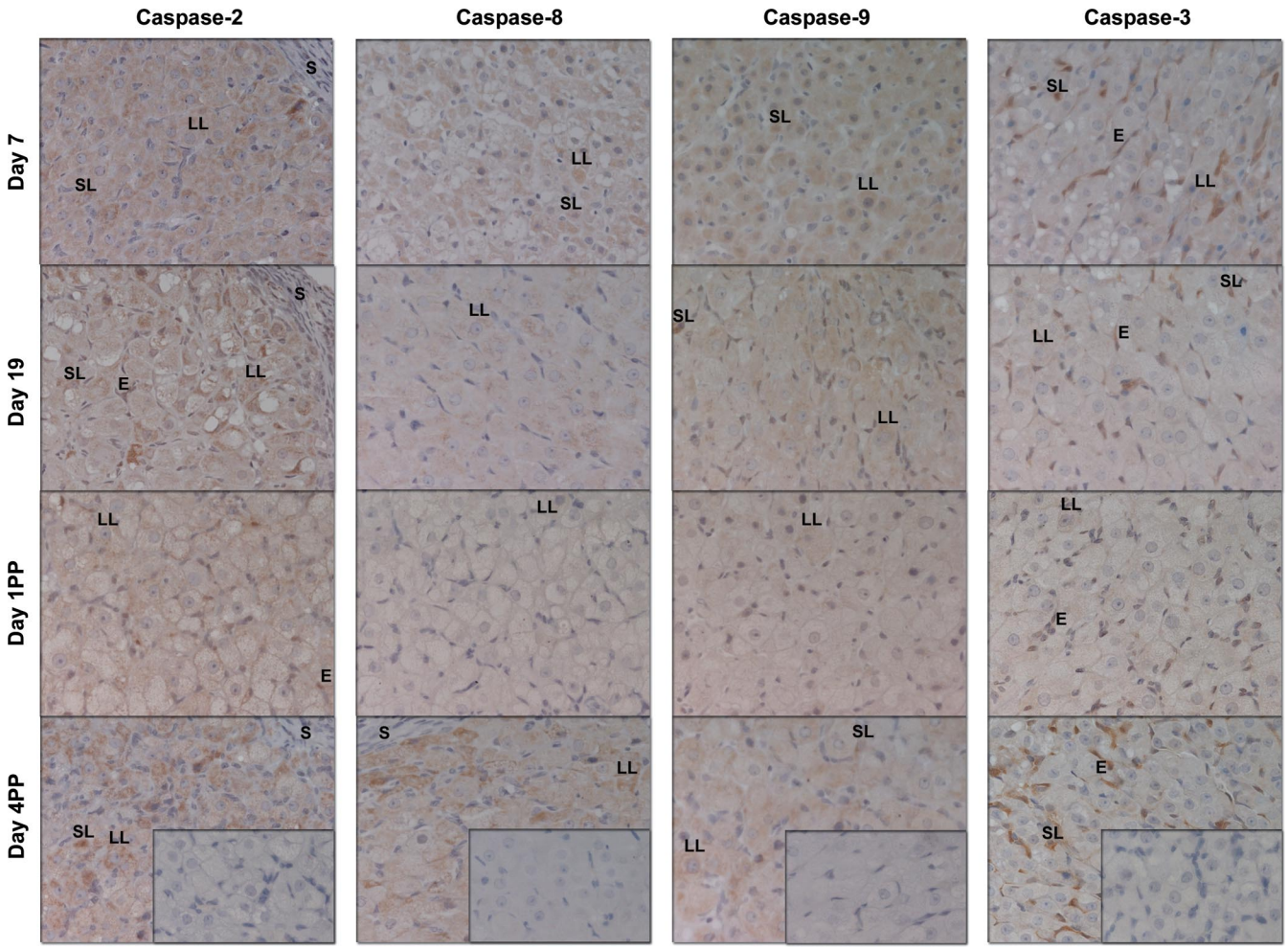

Fig. 3. Immunohistochemistry (IHC) for caspases-2, -8, -9, and -3 in the CL during pregnancy (*days 7* and *19*) and PP (*days 1* and *4*). Caspase-2 IHC: specific immunostaining was found in the cytoplasm and nucleus of large luteal cells (LL), small luteal cells (SL), and endothelial cells (E). Appreciable expression was observed throughout pregnancy and *day 4* PP. Caspase-8 IHC: specific staining was found in the cytoplasm of LL and SL cells. Modest expression was observed during pregnancy. In contrast, immunostaining was minimal at *day 1* PP, followed by an increase at *day 4* PP. Caspase-9 IHC: specific staining during pregnancy and PP showed a similar pattern to caspase-8, except signal intensity appeared greater. Caspase-3 IHC: modest immunostaining was found in the cytoplasm of LL but was notably intense in the nucleus and cytoplasm of many E and SL cells. Specific staining appeared more intense at *days 7* and *19* of pregnancy and *day 4* PP than at *day 21* of pregnancy and *day 1* PP, respectively. No staining was found in the negative controls (e.g., *day 4* PP *insets*) or in the stroma (S).

participates in B-cell activation, whereas caspase-3 may control proliferation and dendritic cell maturation (57). The CL consists of a heterogeneous cell population, including luteal cells (SL and LL), vascular cells (such as endothelial cells), immune cells, and fibroblasts. Immune cells may have functional roles in the CL, and increased numbers of these cells (especially macrophages) are evident during the development and regression of the CL of different species (8, 30, 39, 45, 46, 70). Considerable attention has been given to the potential roles of luteal immune cells in terminating luteal function and assisting in morphological regression of the CL (9). However, monocytes/macrophages are generally not observed in the CL of pregnancy until after parturition (10, 71). These immune cells are known to express the PRL receptor. The apoptotic effect of PRL may be mediated by the immune cells rather than the luteal cells. Furthermore, the invasion of the monocytes/macrophages has been related to the PRL (8, 21).

The IHC and Western blot (caspase-3) analyses indicated that protein (total and/or active fragment) expression of caspases-2, -8, -9, and -3 decreased at *day 1* postpartum. These results are in agreement with the decline in activity of all four caspases following parturition. In contrast, the IHC results suggested an increase in caspase staining by *day 4* postpartum, especially for caspase-3. However, the results were not associated with a rise in total/active caspase-3 as quantitated by Western blot or a rise in the activity of any caspase. The reasons for this discrepancy on *day 4* after delivery are unknown but could be technique related (e.g., the descriptive nature of IHC data and/or differences in antibody interaction with active or inactive caspase in situ vs. blots) or due to differences in regulation of caspase protein vs. enzyme activity in the postpartum interval. Nevertheless, the increase in the number of apoptotic cells after parturition (D1 and D4 postpartum) is out of phase with the peak in the enzyme activity of any of the caspases in the study. These findings suggest the presence of a caspase-independent apoptotic mechanism in the structural regression of the CL after parturition as well. This idea was previously proposed for the rat CL by others (1, 64). However, this mechanism may occur concomitantly with caspase-dependent apoptotic events, since the activity for the

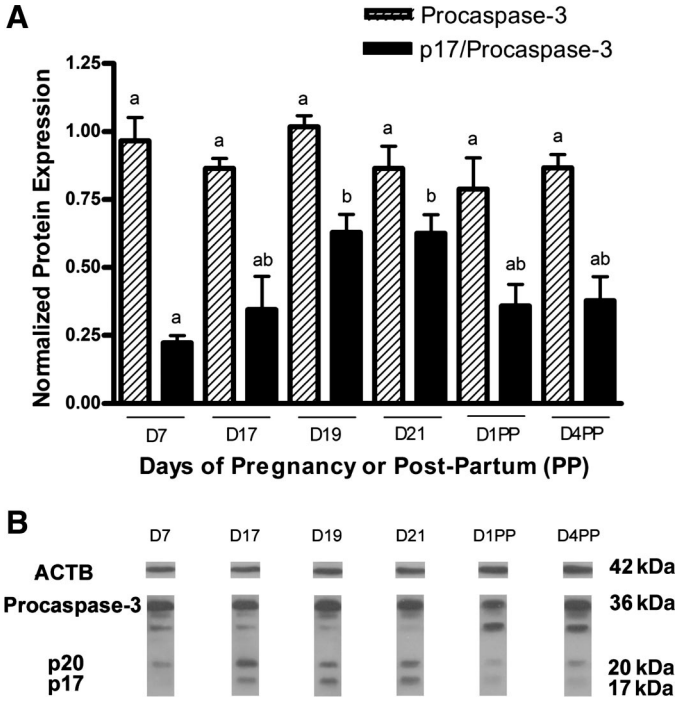

Fig. 4. *A*: analysis of data from Western blots showing the cleavage of caspase-3 in the rat CL throughout pregnancy (*days 7*, *17*, *19*, and *21* of gestation) and PP (*days 1* and *4*). Different letters (a, b) represent a significant difference ($P < 0.05$) in protein levels (means ± SE, normalized to ACTB) between groups ($n = 3$–4/group). *B*: bands corresponding to procaspase-3 (36 kDa), the fragment p20 (20 kDa), the active fragment p17 (17 kDa), and the internal control, ACTB (42 kDa).

four caspases was clearly observed at both *days 1* and *4* postpartum.

PGF2α treatment did not increase the enzyme activity of all three initiators and the main effector caspase until 36 h postinjection. In fact, 16 h post-PGF2α administration, there was a surprising decrease in the caspase-3 activity. The late increase in caspase activities may be an indirect effect of PGF2α, as this hormone interacts with the PGF2α receptor and activates the classical Ca^{2+}-dependent protein kinase (PKC) pathway, which is a rapid signal transduction mechanism (41). It is well known that PGF2α stimulates the mRNA and protein expression of 20α-hydroxysteroid dehydrogenase (20α-HSD) (58, 60) and inhibits the mRNA expression and activity of 3β-HSD (37, 67). However, PGF2α can also reduce the transport of cholesterol by decreasing the expression of sterol carrier protein-2 and steroidogenic acute regulatory protein (StAR) in the rat (14, 38, 55). Progesterone content decreased after PGF2α treatment as expected. This decrease occurred by 24 h postadministration and may be due to the stimulation of 20α-HSD, the inhibition of 3β-HSD expression, or both. Our results suggest that the drop in progesterone precedes and may stimulate the activation of the caspases at 36 h post-PGF2α administration. These findings differ from the results obtained with the pregnancy model, suggesting that the luteolysis induced by the exogenous administration of PGF2α is not necessarily mimicking the physiological luteolysis occurring at the end of pregnancy. This difference could be because of time, dose, or lack of pulsatile secretion of this hormone. Thus this hormone is generally regarded as the primary mediator of functional luteolysis in the rodent CL, but its involvement in morphological regression or structural luteolysis is less clear (16, 36).

Several reports have shown instead the involvement of PRL in the apoptosis/structural luteal regression (8, 22, 32, 35, 43). However, toward the end of pregnancy, the rat CL loses its capacity to respond to PRL. This is due to a decrease in PRL receptor expression (66) and to an increase in SOCS3, which prevent PRL receptor signaling (15). This evidence indicates that PRL may not play a key role in the initiation of luteolysis. Since PRL receptor expression is recovered after parturition, PRL participation in structural regression of the postpartum CL is assured. In addition, the preovulatory PRL surge in the evening of proestrus is presumed to initiate the regression of the CL from the preceding cycle (8, 20, 22). However, it is also

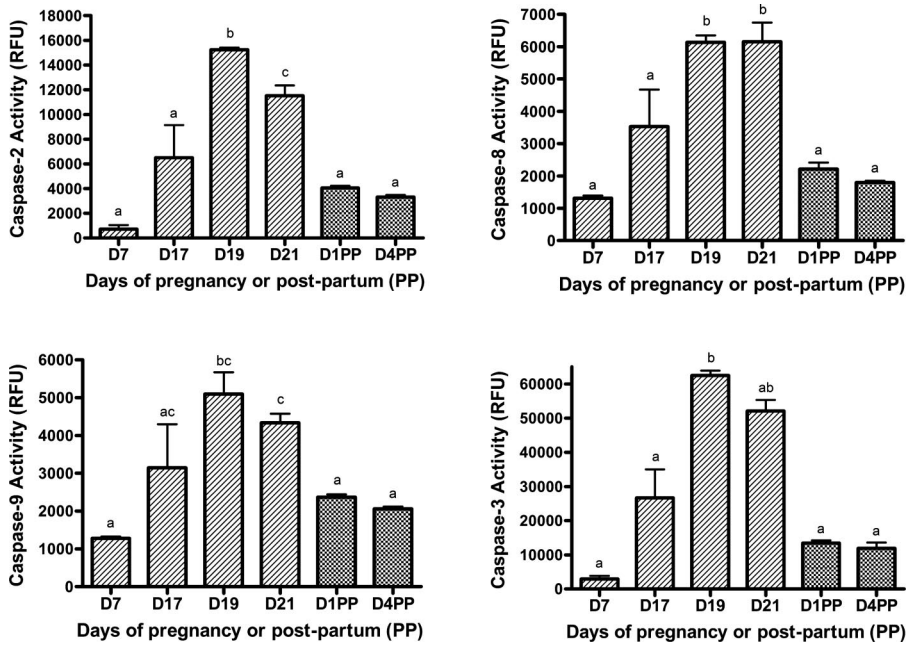

Fig. 5. Enzyme activity levels (means ± SE) for the initiator caspases (caspases-2, -8, and -9) and the effector caspase (caspase-3) in the rat CL during pregnancy (*left*: *days 7*, *17*, *19*, and *21* of gestation) and PP (*right*: *days 1* and *4*). Activity is expressed in relative fluorescence units (RFU). Different letters (a, b, c) represent a significant difference ($P < 0.05$) in the activity between time points ($n = 3$–4/group).

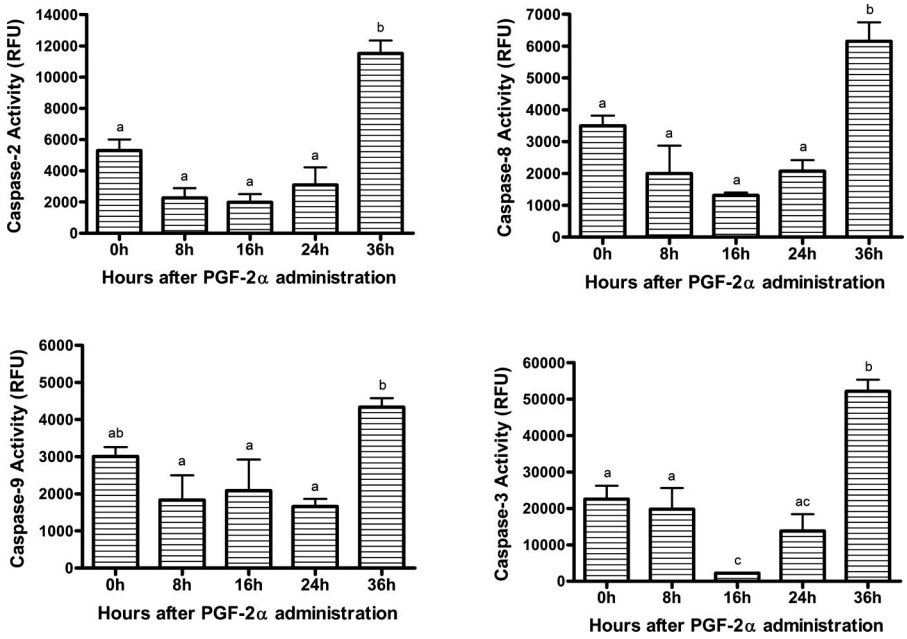

Fig. 6. Enzyme activity levels (means ± SE) for the initiator caspases (caspases-2, -8, and -9) and the effector caspase (caspase-3) in the rat CL after PGF2α administration (0, 8, 16, 24, and 36 h). Different letters (a, b, c) represent a significant difference ($P < 0.05$) in the activity between groups ($n = 3–4$/group).

well known that, in lactating rats, PRL has a luteotropic effect on luteal cells (26), suggesting a dual effect of this hormone on this cell type.

Interestingly, in the present study, the highest levels of caspase activity were detected at *days 19–21* of gestation, when functional luteolysis was just beginning. In addition, PGF2α administration to pregnant rats increased the activity of all four caspases. Several reports indicate that PGF2α treatment increased caspase expression and/or activity in different species, such as mouse, rat, cow, and sheep (53, 73). These findings suggest an important role of the PGF2α in caspase activation and probably in apoptosis of the luteal cells. Supporting this idea, Yadav et al. (73) demonstrated in a pseudopregnant rat model the importance of the extrinsic apoptotic signaling cascade and the activation of caspases-8 and -3 in mediating luteal tissue apoptosis after PGF2α treatment. Carambula et al. (12) also hypothesized that PGF2α initiates luteolysis in vivo, at least in part, by increasing the bioactivity or bioavailability of cytokines such as Fas-L that then activate caspase-3, triggering apoptosis of the mouse luteal cells. Nevertheless, further studies on the involvement of PGF2α, caspases, and apoptosis in luteolysis are warranted.

Caspase-2 can link both apoptotic pathways (the extrinsic death receptor and the intrinsic mitochondrial pathways) through cleavage of a member of the Bcl-2 family, Bid (28). Therefore, physiological luteal regression may involve the cooperation of both extrinsic and intrinsic apoptotic pathways. Several studies demonstrate the presence of the extrinsic pathway in luteal cells (1, 12, 32, 73), although there are also data supporting the involvement of the intrinsic mitochondrial pathway in luteal cell death (25, 44).

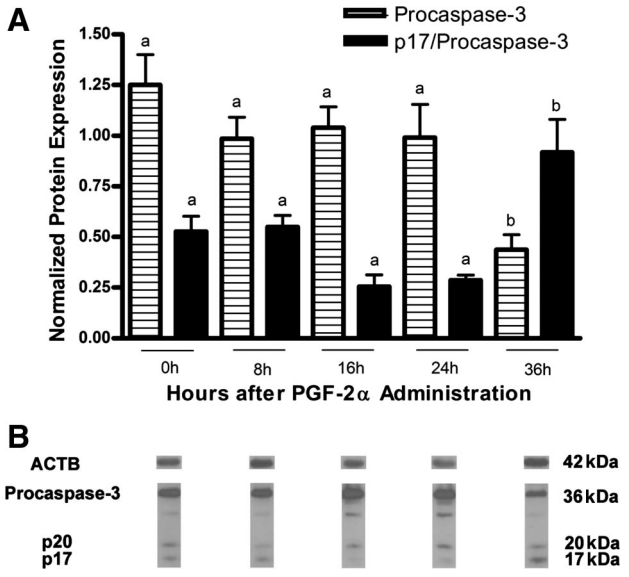

Fig. 7. *A*: analysis of data from Western blots showing the cleavage of caspase-3 in the rat CL at different times (0, 8, 16, 24, and 36 h) after PGF2α administration. Different letters (a, b) represent a significant difference ($P < 0.05$) in protein levels (means ± SE) between groups ($n = 3–4$/group). *B*: bands corresponding to procaspase-3 (36 kDa), the fragment p20 (20 kDa), the active fragment p17 (17 kDa), and the internal control (ACTB, 42 kDa).

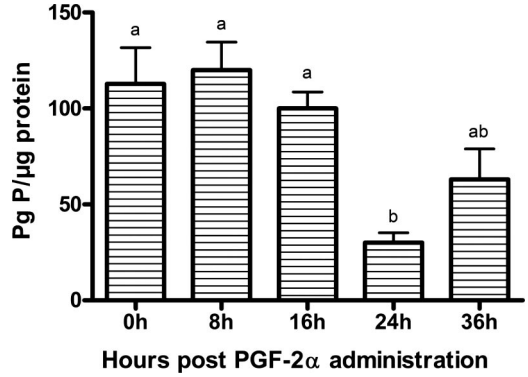

Fig. 8. Progesterone levels (means ± SE) at different times (0, 8, 16, 24, and 36 h) after PGF2α administration at *day 14* of pregnancy ($n = 3–4$/group). Different letters over SE bars indicate significant differences ($P < 0.05$) between time points.

In summary, our findings suggest that *1*) an increase in the activity of caspases-2, -8, -9, and -3 is associated with the early events of functional luteolysis (at the end of pregnancy) in the rat, and *2*) PGF2α plays an important role in the regulation of the activity of several members of the caspase family in the rat CL. In addition, the control of protein expression of the caspases in the CL during pregnancy and postpartum may differ from the activity of these proteases.

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