Expression of caspase-2, -3, -8 and -9 proteins and enzyme activity in the corpus luteum of the rat at different stages during the natural estrous cycle

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Abstract

Apoptosis is associated with the regression of the corpus luteum (CL) in many species. Since caspases play a central role in apoptosis, we studied several initiators (-2, -8, and -9) and the main effector (-3) caspase in the CL during the estrous cycle of the rat. Two different populations of CL (old and new) were identified on ovaries at estrus and diestrus II (DII). Diminished ($P<0.05$) luteal progesterone content and P450scc levels suggested that functional luteolysis occurred between the new CL at DII and old CL at estrus, whereas the decline ($P<0.05$) in luteal weight indicated that structural regression was occurring between old CL at estrus to DII. Immunostaining for caspase-2 in luteal and endothelial cells appeared to increase as the luteal phase progressed, peaking at DII in the old CL. However, caspase-8 and -9 immunostaining showed little change with a slight increase at estrus in the old population. Notably, caspase-3 staining appeared to peak at DII in the new CL. Enzyme activity of caspase-9 increased ($P<0.05$) in the new CL at DII, followed by that of caspase-2 and -3 in old CL at estrus. Caspase-8 activity did not change at any stage. The number of apoptotic cells increased at DII in the old CL. These results suggest an important role for this protease family during early events of luteolysis in the rat estrous cycle.
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Introduction

The corpus luteum (CL) is a transient endocrine gland derived from the wall of the ovarian follicle after ovulation that produces progesterone, the hormone necessary for the establishment and maintenance of intrauterine pregnancy in mammals (Bowen-Shauver & Gibori 2004, Stouffer 2004). In the absence of pregnancy at the end of each ovarian cycle, or when it is no longer required for the maintenance of pregnancy, the CL ceases to produce progesterone and regresses in a process called luteolysis. The demise of the CL occurs in two phases. The first phase consists of the loss of the ability to produce progesterone and it is defined as 'functional luteolysis'. The second phase is the 'structural luteolysis' or complete morphological regression of the CL. In rats, structural luteolysis is a slow process and the CL may remain in the ovary throughout several estrous cycles before its complete dissolution (Bowen & Keyes 2000). In rodents, prostaglandin F2α (PGF-2α) is a uterine-derived factor that initiates luteolysis. In addition, the preovulatory prolactin (PRL) surge in the evening of proestrus is presumed to initiate the regression of the CL from the preceding cycle (Bowen et al. 1996, Gaytan et al. 1998, 2000). However, it is also well known that in lactating rats PRL has a luteotropic effect on luteal cells (Goyeneche et al. 2003), suggesting a dual effect of this hormone on this cell type. Considerable progress has occurred in elucidating the role of PRL and PGF-2α in controlling luteal regression, but the molecular and cellular mechanisms whereby these signals cause luteolysis are poorly understood. Further knowledge of the molecular mechanisms that regulate CL regression could contribute to the understanding and treatment of luteal dysfunction associated with infertility. Considerable evidence suggests that programmed cell death, or apoptosis, is associated with luteal regression in many species, including rodents and domestic animals (Sawyer et al. 1990, Juengel et al. 1993, Bacci et al. 1996, Hasumoto et al. 1997,

Goodman *et al.* 1998, Guo *et al.* 1998), but a causal role has not been established.

Apoptosis is a physiological process that plays a critical role in the maintenance of homeostasis in multicellular organisms. Studies in various model systems (Tilly *et al.* 2004), indicate that signaling for apoptosis occurs through multiple independent pathways that converge in a common machinery of cell destruction. Members of the caspase family are cysteine aspartic acid-specific proteases involved in either the early or the final apoptotic events, and the activation of these proteases is a pivotal event in apoptosis (Martin & Green 1995, Alnemri *et al.* 1996, Guo *et al.* 1998, Nicholson 1999). The caspase family includes two main categories: initiators and effectors (Hasumoto *et al.* 1997, Goodman *et al.* 1998). Initiator caspases have long pro-domains and play a role upstream of the cell death cascade through the activation of the effector caspases. In contrast, the effector caspases possess short pro-domains and are mainly responsible for the cleavage of key apoptotic substrates, such as cell-cycle signaling molecules, DNA repair enzymes, mRNA-processing components, cytoskeletal and nuclear scaffold proteins, and nuclease activator factors (Sawyer *et al.* 1990, Juengel *et al.* 1993, Bacci *et al.* 1996, Goodman *et al.* 1998). It is recognized that caspase-3 is a key effector and it is common to both the mitochondria and the death receptor pathway (Bacci *et al.* 1996, Boone & Tsang 1998, Goodman *et al.* 1998, Davis & Rueda 2002).

Several studies indicate that members of the caspase family are expressed in ovarian follicular cells (Dimmeler *et al.* 1997, Weiland *et al.* 2000, Villavicencio *et al.* 2002); however, there are few studies to date on caspase expression or activity in the CL (Boone & Tsang 1998, Rueda *et al.* 1999, Davis & Rueda 2002). Recent evidence suggests that caspase-3 is expressed in human luteinized granulosa cells (Khan *et al.* 2000) and the CL (Krajewska *et al.* 1997, Krajewski *et al.* 1997). Also, there are data from caspase-3-deficient mice (Carambula *et al.* 2002) suggesting that this enzyme is pivotal for the timely regression of the CL.

We hypothesized that if apoptosis plays a key role in the tissue remodeling associated with the regression of the rodent CL, then caspase protein expression and/or enzyme activity would increase during luteolysis in the natural estrous cycle of the rat. In these initial studies, we focused our research on the two different population of the CL at estrous and diestrous II stages during the natural estrous cycle of the rat. We studied different caspases: one initiator (-9) involved in mitochondrial-initiated apoptosis, one initiator (-8) involved in death receptor-initiated apoptosis as well as one initiator caspase (-2) related with both pathways, and also the main downstream effector, caspase-3 (Tilly *et al.* 2004). In addition, we measure morphological features of apoptosis and steroidogenic patterns in the diverse population of the CLs in study.

Materials and Methods

Animals and protocols

Adult female Sprague–Dawley rats, were allowed food and water *ad libitum* and kept at room temperature in a range of 21–23 °C in a 12 h light:12 h darkness cycle. Animals were sacrificed by CO_2 asphyxiation. The ovaries were removed and cleaned prior to subsequent assays. All experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approval of the OHSU IACUC and the IBYME ethical committee was obtained. Estrous cycling stages were determined by daily examination of vaginal cytology, and animals demonstrating at least two consecutive 4-day cycles were used for experiments. Ovaries were collected in estrus (E) and diestrus II (DII). Since rodent CL exists for more than one cycle, we decided to study caspase expression and activity in two populations of CL present on the ovaries at estrus and DII ($n=6$–7 per time group). We selected the estrous stage since the recent surge of PRL triggers luteolysis of the CL from the previous cycle, but not of the recently formed CL. In addition, the diestrous II stage was chosen to study the most recent CL prior to function (pregnancy) as well as the regressed CL from the previous cycle. The pattern of progesterone secretion during the estrous cycle consists of two major increases, during DII and on the afternoon of proestrus. Progesterone secretion begins on the afternoon of diestrus I and reaches peak values by early morning of DII, followed by a drop. Progesterone levels remain low until the afternoon of proestrus when a surge occurs, which is coincident with the onset and duration of the LH surge (Smith *et al.* 1975). Thus, samples included CL at formation, prior to its function (new CL at estrus), prepared for an eventual pregnancy (new CL at DII), during functional regression (old CL at estrus) and when structural regression begins (old CL at DII).

CLs were dissected from the isolated ovaries (estrus and DII groups) under a stereoscopic microscope as previously described in our laboratory (Andreu *et al.* 1998). Two types of CLs were easily recognized under the microscope in both groups. Old and new CLs were identified and collected separately based on vascularization and size criteria in accordance to the CL stage. The old ones were whitish and contained thin veins, whereas the new ones appeared red and had thicker veins. A representative group of old and new CLs were weighed to confirm a difference in mass between groups. Pools of old and new CLs from one ovary from each rat were frozen in liquid nitrogen and stored at −80 °C for protein isolation. Contralateral ovaries ($n=6$–7 per stage) were fixed in formalin for immunohistochemical analysis of caspase-2, -3, -8, and -9 as well as apoptosis analysis.

A group of rats were mated overnight as a control of CL rescue. The CLs were isolated under a stereoscopic

microscope and frozen in liquid nitrogen for protein isolation and measurement of caspase activities. Day 0 of gestation was designated the morning on which spermatozoa were found in a vaginal smear and rats from day 7 of pregnancy were used.

Luteal progesterone levels

Steroids were extracted as previously described in our laboratory (Saragüeta *et al.* 1989). Old and new CLs at estrus and DII (three to five CLs per group) were homogenized in acetone with Ultra-Turrax (IKA Werk, Breisgau, Germany). Known quantities of labeled progesterone (^{3}H-progesterone) in acetone (approximately 3000 c.p.m.), were added to each sample as internal standard. An aliquot was taken from each homogenate for protein measurement. After complete homogenization, the samples were centrifuged (1600 *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 adding distilled water, the samples were submitted to a solvent partition with *n*-hexane and dichloromethane, and the upper layer was discarded and the lower phase evaporated. Finally, samples were stored in distilled water for later analysis by RIA.

Caspase-2, -3, -8, and -9 immunohistochemistry (IHC)

Ovaries were fixed in 10% neutral buffered formalin for 1 week. 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. Endogenous peroxidases were then quenched with a 25-min incubation in 3% H_2O_2. Sections were incubated in PBS prior to antigen retrieval in 0.01 M citrate. Sections were placed in a blocking buffer (2% BSA in PBS) for 20 min, and then incubated with the primary antibody in 1% BSA PBS buffer for 1 h at room temperature and overnight at 4 °C. Concentrations for caspase-2 (sc-626; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), -3 (sc-7148; Santa Cruz Biotechnology, Inc.), -8 (3020-100, Biovision Research Product, Mountain View, CA, USA), -9 (sc-7885; Santa Cruz Biotechnology, Inc.), and antibodies were 1:500 (-2), 1:1000 (-3), 1:200 (-8 and -9) respectively. According to the manufacturer, these antibodies recognize both the inactive as well as the active form of the caspase. Primary antibody was detected using a biotinylated anti-rabbit IgG secondary antibody (1:400; Vector Laboratories, Burlingame, CA, USA) and the Vector ABC-Elite Kit (Vector Laboratories); diaminobenzidine with a metal-enhanced substrate (Cat #1718096, Roche, Oenzberg, Germany), and counterstained with hematoxylin. For each caspase examined, negative controls lacking primary antibody were processed on adjacent tissue sections.

Caspase-2, -3, -8, and -9 activity assays

From each ovary (*n* = 4–5), a pool of old or new CLs per stage was used to evaluate the enzyme activity. Also, isolated CLs from day 7 of pregnancy were evaluated. A fluorometric assay kit (630225; Clontech), which contains fluorogenic substrates specific for different caspases (-2, -3, -8, and -9), immobilized in separate wells, was used as previously described (Peluffo *et al.* 2005). Fifteen micrograms of the extracted protein from each pool of CLs in homogenization buffer (50 mM Tris–HCl, 150 mM NaCl, 10% glycerin, and 1% Triton X-100) containing protease inhibitors (Halt Protease Inhibitor Cocktail Kit, EDTA free; Pierce Biotechnology, Rockford, IL, USA) were added to the wells. The plate was incubated in a fluorescence plate reader at 37 °C for 3 h and fluorescence was read every 10 min. The activity was determined by fluorometric detection (Ex: 380 nm, Em: 460 nm) and negative controls (blank, without sample) were subtracted from all samples. Results at 2 h were selected, as the manufacturer suggested. Baseline values of negative controls and samples with specific inhibitors did not increase during the 2 h interval.

Western blotting

After boiling for 5 min, 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. Non-specific binding sites were blocked overnight in Tris-buffered saline (4 mM Tris-Cl (pH 7.5), 100 mM NaCl) containing low-fat powdered milk (5%) and Tween 20 (0.5%) at 4 °C. The membranes were incubated overnight with rabbit polyclonal anti-P450scc (gift from Dr Anita H Payne, Stanford University Medical Center, Stanford, CA, USA) and anti-caspase-3 (sc-7148; Santa Cruz Biotechnology, Inc.) in a concentration of 1:2000 and 1:200 respectively. Protein bands were visualized by incubating the blots for 1 h with peroxidase-conjugated secondary anti-rabbit IgG (1:1000, Cat # A4914 Sigma Chemical Co.). Finally, the membranes were then incubated with Western Lightning Chemiluminescence Reagent Plus (cat # NEL104; Perkin-Elmer Life Sciences, Inc., Boston, MA, USA) for 1 min and exposed to X-ray film in a dark room. Protein expression was quantified by densitometric analysis using Scion Image Software for Windows (Scion Corporation, Worman's Mill, CT, USA). Consistency of protein loading was evaluated by staining the membranes with Ponceau-S and also the density in

each specific band was normalized to the density of different internal controls depending on the size of the bands. GAPDH was used to normalize the P450scc data and β-actin was used as an internal control for the caspase-3 data.

Apoptotic cell analysis (TUNEL)

Ovaries ($n=6-7$) from rats in estrus or DII were fixed in 10% neutral buffered formalin for 1 week and were paraffin-embedded. Serial 4 μm sections were mounted for TUNEL assays. Nuclear DNA fragmentation in luteal cells was detected using the DeadEnd Colorimetric TUNEL System (G7130; Promega), following the manufacturer's instructions with minor modifications as we previously described (Peluffo *et al.* 2005). A microscope with a 100× objective was used and three randomly selected fields were analyzed from each CL section (three old and three new CL per section/ovary, six to seven ovaries per stage). Results were expressed as percentage of apoptotic cells.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test for paired data (new versus old, CL, within stages) or the ANOVA Test for comparison between multiple data (CL between stages), followed by Student–Newman–Keuls method or Dunn's test using the Sigma Stat software package (SPSS, Chicago, IL, USA). Differences were considered significant at $P<0.05$.

Results

Indices of luteal structure–function

Corpus luteum wet weight, progesterone levels, and P450ssc content

Weight of the new and old CLs at both estrus and DII revealed significant differences (new versus old CL at estrus: 3.9 ± 0.4 vs 11.3 ± 0.7 mg; DII: 12.2 ± 0.6 vs 5.2 ± 0.4 mg; $P<0.05$). Notably, differences between weights at the different CL stages (estrus and DII) confirmed the presence of the two populations of CLs dissected based on vascularization and size criteria. When the data are analyzed as a function of advancing CL age (Fig. 1A), CL weight increased in the new CL from estrus to DII followed by a decrease in the old CL from estrus to DII.

When progesterone content was measured in the various CL stages, a significant increase ($P<0.01$) was observed in the new CL at the diestrous II stage in comparison to the other stages (Fig. 1B).

Western blotting for P450ssc revealed a band of 53 kDa in all samples studied (Fig. 1C). When normalized to GAPDH (Glyseraldehyde-3-phosphate dehydrogenase) there was a significant increase ($P<0.05$) in the P450ssc protein levels at DII in the new CL compared to all other groups (Fig. 1C).
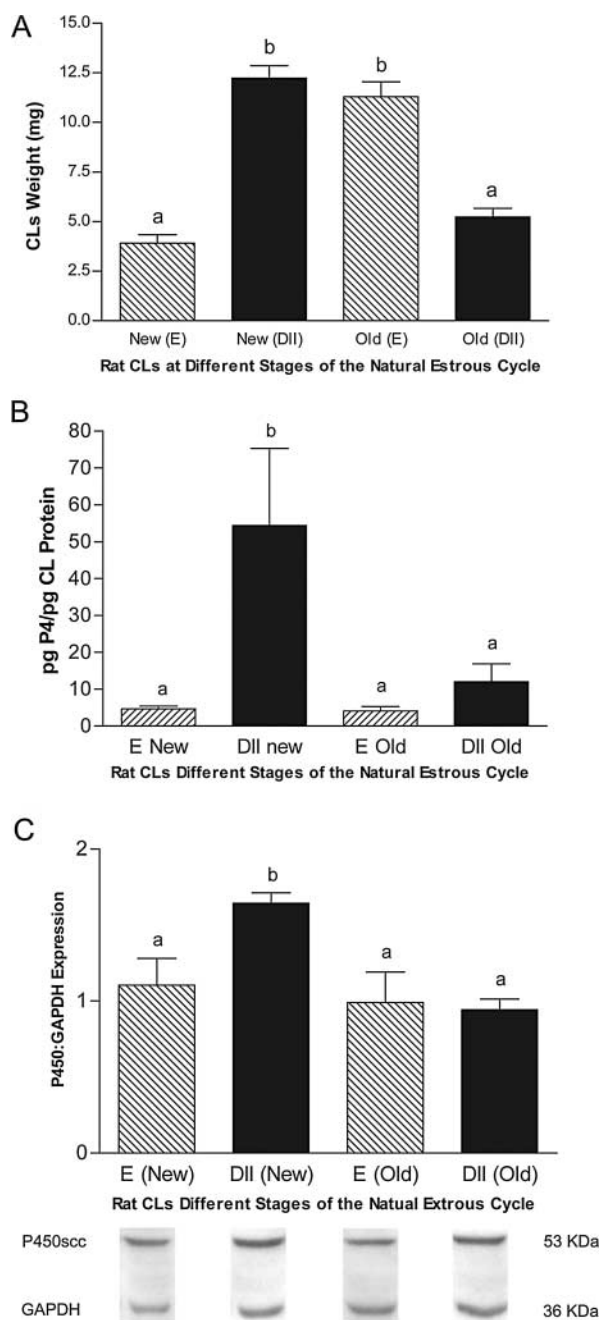

Figure 1 (A) Weight (mean±s.e.m.) of the old and new CL of rats at estrus (E) and diestrus II (DII) of the natural estrous cycle. Bars with different letters (a, b) represent a significant difference ($P<0.05$) between groups ($n=12-15$ per group). There was a significant difference between the old and new CLs within each stage, between new CL across stages and between old CL across stages. (B) Progesterone levels in the new and old CL at estrous (E) and diestrous II (DII) stage of the rat ($n=3-5$ per group). 'b' represents a significant increase ($P<0.01$) in progesterone levels of the new CL at DII and the other CL stages 'a' studied. (C) Western blot of P450ssc during the natural estrous cycle of the rat ($n=3-4$ per group). Different letters (a and b) represent a significant difference ($P<0.05$) in P450ssc protein levels between the new CL at diestrous II stage and other CL stages. The bottom section illustrates gels with the bands corresponding to P450ssc (53 kDa) and the internal control, GAPDH (36 kDa).

Analysis of caspase-2, -3, -8, and -9 IHC

Immunolabeling for the initiator caspase-2, -8, and -9 (Fig. 2) was observed in the CL at all stages in study. 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 (END), but not in the surrounding stroma. At estrus and DII, the old CLs displayed more intense staining for caspase-2 in the cytoplasm and nucleus of all types of cells described.

In contrast, specific immunostaining for caspase-8 and -9 (Fig. 2) displayed little change in expression in the large luteal cells, while no staining was detected in the endothelial or in the SL cells.

Immunostaining for the main effector caspase-3 (Fig. 2) was also detected in the CL at all stages. Immunoreactivity for caspase-3 was also found in the luteal cells (SL and LL) and endothelial cells (END), but no staining was found in the stroma. Interestingly,
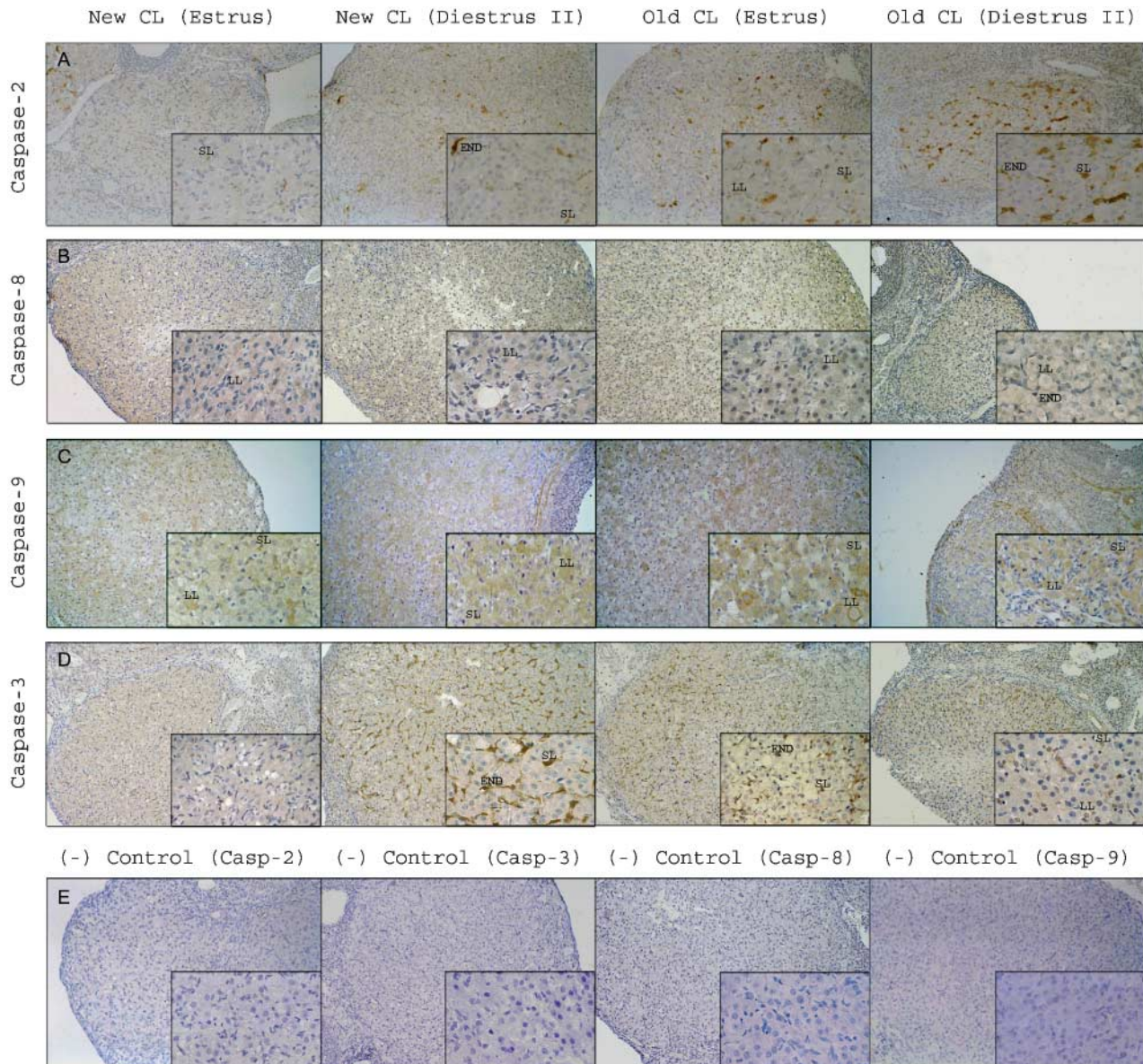

Figure 2 Immunohistochemistry for caspase-2, -8, -9, and -3, in the rat CL at estrus (E) and DII of the natural estrous cycle (left to right panels new (E), new (DII), old (E), and old (DII)). (A) Immunostaining for caspase-2 appeared to increase as the luteal phase progressed, peaking at DII in the old CLs. Specific staining for this caspase was found in the cytoplasm and nucleus of the small (SL) and the large (LL) luteal cells as well as in the endothelial cells (END), but not in the surrounding stroma (S). However, (D) caspase-3 protein staining appeared to peak at DII but in the new CLs. Specific staining for this caspase was also found in the nucleus and cytoplasm of SL, LL, and END, but no staining was found in the stroma. Immunolabeling for caspase-8 and -9 (B and C respectively) was found in the cytoplasm of the luteal cells, showing a little change in expression with a slight increase at estrus in the old CLs. Negative controls without the primary antibody showed no staining in any of the four IHC assays. These controls for caspase-2, -8, -9, and -3 are shown in increasing order (E).

staining for the effector caspase-3 was also localized in the nucleus in addition to the cytoplasm. Notably, immunolabeling appeared to peak at DII of the new CLs.

Negative controls for the four caspases, without the primary antibody, showed no staining (Fig. 2).

Caspase activity assays

Levels of caspase-8 enzyme activity did not change between stages (Fig. 3B), whereas caspase-2 activity (Fig. 3A) peaked in the old CL at estrus (at the beginning of regression), and caspase-9 (Fig. 3C) increased in the new CL at DII remaining high in the old CL. The activity levels of the main effector caspase-3 (Fig. 3D) peaked in the old CL at estrus similar to the caspase-2 results. In old CL at estrus, caspase-2, -9, and -3 activity levels were 7.6-, 1.4-, and 1.7-fold greater ($P<0.05$) respectively compared to new CL. Moreover, only caspase-9 activity in old and new CL at DII was significantly higher (1.6- and 1.96-fold greater respectively) than the activity in the new CL at estrus.

Notably, activity levels for the four caspases in the CL at day 7 of pregnancy were eight- to tenfold lower, in comparison to the old CL from the estrous cycle (2965 ± 832.1 and $56\,440 \pm 16\,420$ respectively, $P<0.01$, data not shown).

Analysis of western blotting of caspase-3

Western blot analysis revealed two different bands, corresponding to the pro-caspase (33 kDa) and to the active fragment p17 (17 kDa; Fig. 4C). When data were normalized to β-actin levels, higher pro-caspase levels were present in the new CL at estrus and in the old CL at DII (Fig. 4A). When the expression of the active fragment was determined in relationship to the pro-caspase, the ratio was significantly increased in the new CL from estrus to DII, and declined in the old CL (Fig. 4B).

TUNEL

Nuclear DNA fragmentation was detected in the rat CL by TUNEL assay at estrus and DII stages of the natural estrous cycle (Fig. 5A). Cells undergoing apoptosis were expressed as the percentage of apoptotic cells in each stage. There was an increase ($P<0.001$) in the
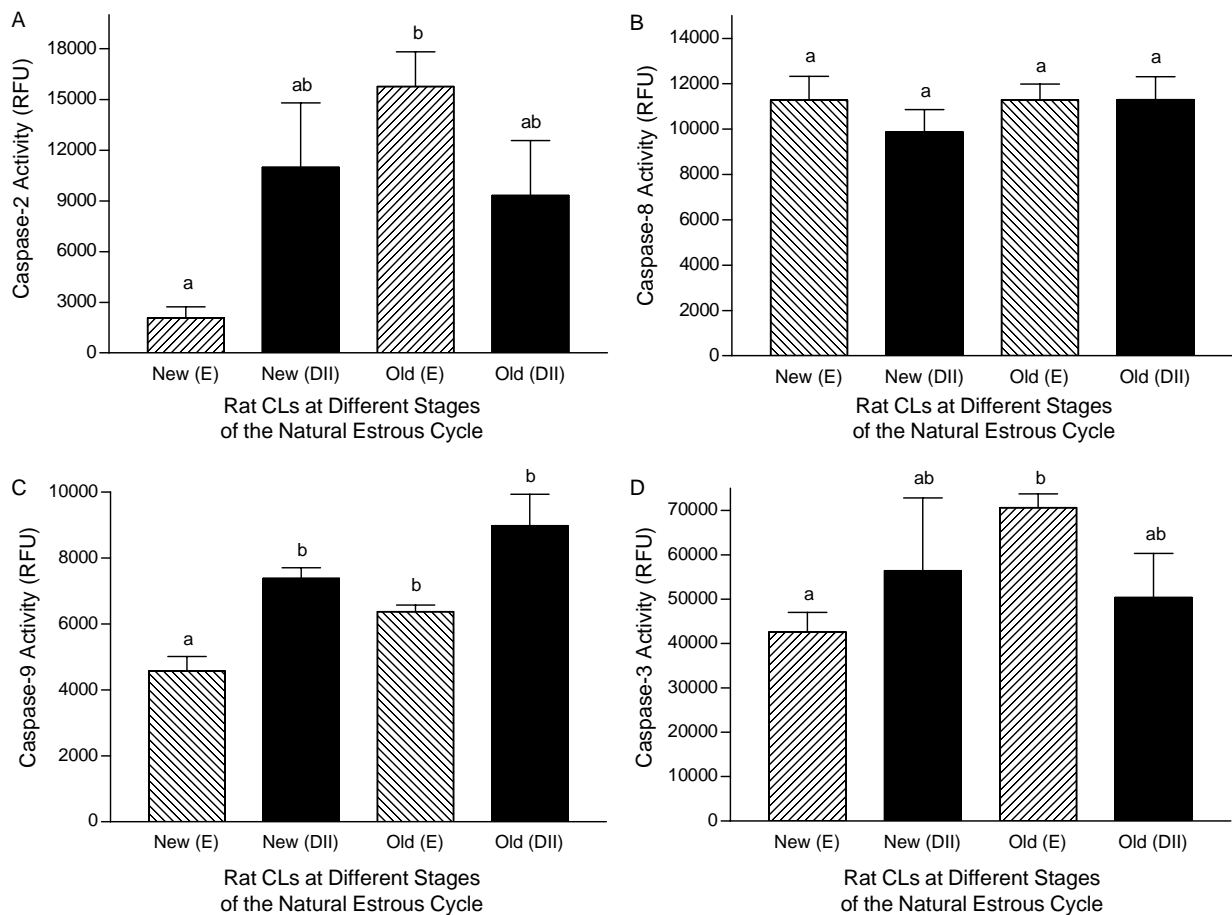

Figure 3 Enzyme activity levels (mean + S.E.M.) for initiator caspases (-2 (A); -8 (B); -9 (C)) and the effector caspase-3 (D) in the rat CL at estrus (E) and DII. Bars with different letters (a and b) represent a significant difference ($P<0.05$) in the activity between groups ($n=4–5$ per group). The activity is expressed in relative fluorescence units.
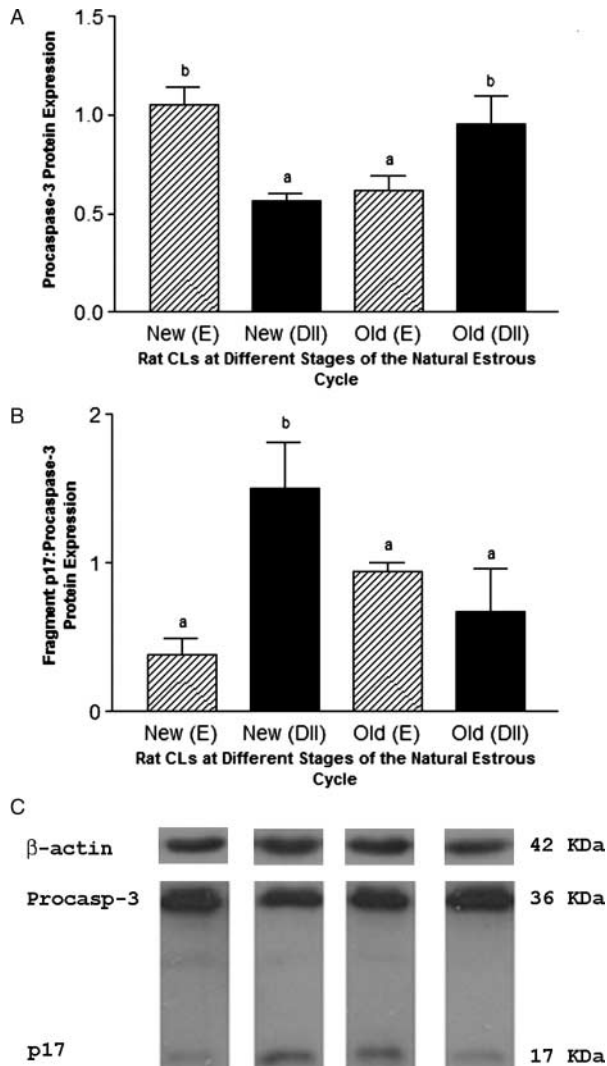
Figure 4 Cleavage of caspase-3 in the rat CL at estrus (E) and DII measured by western blot. Bars with different letters (a and b) represent a significant difference ($P<0.05$) in protein levels between groups ($n=$ 3–4 per group). (A) Levels of the pro-caspase, normalized to β-actin, showed higher levels ($P<0.05$) at estrus of the new CL as well as at DII of the old CL in comparison with the other CL stages. (B) The ratio of the active fragment p17 to the pro-caspase was significantly higher ($P<0.05$) at DII in the new CL. (C) The bands corresponding to the pro-caspase-3 (33 kDa), the active fragment p17 (17 KDa) and the internal control, β-actin (42 kDa).

percentage of apoptotic cells in the old CLs at estrus and DII (Fig. 5B), as luteolysis progressed.

Moreover, CL sections stained with hematoxylin–eosin were used to count apoptotic cells at estrus and DII stages following a validated morphologic procedure (Telleria *et al.* 2001), considering only luteal cells with advanced signs of apoptosis (i.e., containing multiple nuclear fragments or small densely stained nucleus). Similar results were obtained using this procedure (data not shown), as compared to the TUNEL assay.

Discussion

This study provides the first evidence on dynamic protein expression and activity of multiple caspases in the CL during the natural estrous cycle of the rat, and their relationship with luteal structure-function (i.e., CL weight, progesterone content, P450scc levels).

IHC analysis for the initiator caspase-2, -8, -9 and the effector caspase-3 showed different patterns of expression for these members of the caspase family. Concerning caspase-8 and -9 immunostaining, low expression was found for these proteases with little changes in the large luteal cells, while no staining was detected in the endothelial or in the small luteal cells. However, caspase-2 expression appeared to increase as the luteal phase progressed (from the new CL at estrus to the old CL at DII). Specific staining was found in the cytoplasm as well as in the nucleus of luteal (SL and LL) and endothelial cells. However, staining in the large luteal cells was mainly in the cytoplasm and much lighter than in the endothelial and the small luteal cells. These types of cells may have different regulatory mechanisms of cell death and the high expression of this protease suggests an important role of caspase-2 in the apoptotic death of these cells. Several studies demonstrated apoptosis of endothelial cells and suggested its activation through the tumor necrosis factor-α pathway (Friedman *et al.* 2000, Pru *et al.* 2003). Moreover, even though caspase-2 was the first mammalian apoptotic caspase identified (Kumar *et al.* 1994, Wang *et al.* 1994), little is known about its function. Some authors associate it to the death receptor pathway (Ahmad *et al.* 1997, Duan & Dixit 1997), whereas others relate it to the mitochondria pathway (Guo *et al.* 2002, Robertson *et al.* 2002). Besides, Bergeron *et al.* (1998) demonstrated in a caspase-2 knockout mice model, an excess number of germ cells endowed in the ovaries and an increase in the cell death of motor neurons; suggesting that caspase-2 could act both as a positive and as a negative cell death effector, depending upon cell lineage and stage development (Bergeron *et al.* 1998). It is known that initiator caspases are activated following direct association with death domain proteins. Interestingly, immune cells as well as cytokines were shown to play a role in regulating luteal function in rats, mice, cows, and women (Brannstrom & Friden 1997, Terranova & Rice 1997, Friedman *et al.* 2000). Moreover, during luteolysis, macrophages are attracted and infiltrate in the CL, increasing the local production of cytokines (Brannstrom *et al.* 1994). Thus, this increase in local cytokines may trigger apoptosis in the rat CL through the activation of the caspase-2 and the death receptor pathway; however, further studies are warranted to prove this hypothesis.

Notably, immunostaining for the effector caspase-3 was also found in the cytoplasm as well as in the nucleus of the luteal (SL and LL) and the endothelial cells; and
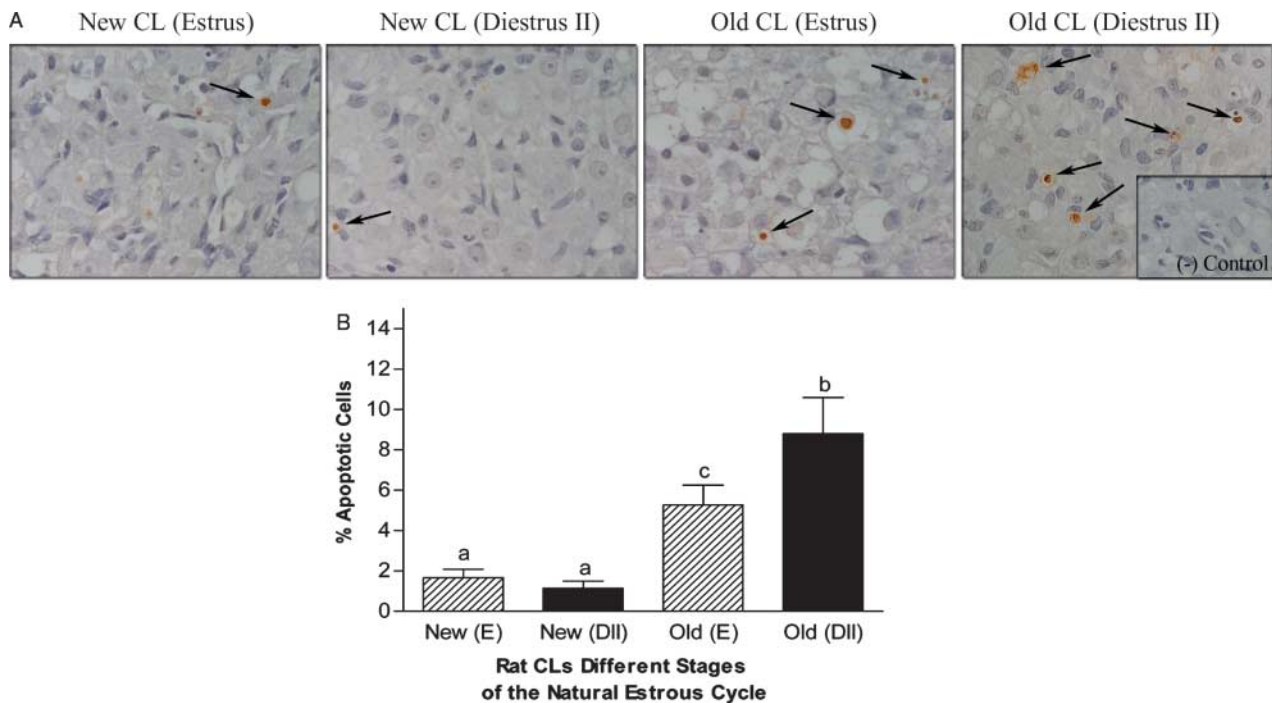
Figure 5 Sections of old and new CL at estrus (E) and (A) DII during the natural estrous cycle of the rat, showing some advanced signs of apoptosis as positive staining for the TUNEL assay (arrows). (B) TUNEL analysis revealed an increase ($P<0.05$) in the number of apoptotic cells at estrus and DII of the old CLs, as luteolysis progressed. Different letters (a and b) represent a significant difference ($P<0.05$) between the old CL at estrous and diestrous II stage and other CL stages. Three randomly selected fields were analyzed from each CL section (three old and three new CL per section/ovary, six to seven ovaries per group (estrus or DII)). Apoptotic index is expressed in percentage.

specific staining was more intense in the endothelial and small luteal cells. Nuclear staining appeared in the new CL at DII and remained in the old CL at the estrous and diestrous II stages. In addition, initiator caspase-2 immunostaining appeared to peak in the new CL at DII, prior to the peak seen for the caspase-3 activity in old CL at estrus, in accordance to our results in the rhesus monkey CL (Peluffo *et al.* 2005). Moreover, western results also showed an increase in the cleavage of caspase-3 in the new CL at the diestrous II stage. Significantly, caspases are found as an inactive form in different intracellular compartments, but after activation these proteases (in particular the effectors, e.g., caspase-3) are translocated to specific sites such as the nucleus where they can cleave specific target proteins (Porter 1999, Susin *et al.* 1999, Zhivotovsky *et al.* 1999, Tilly *et al.* 2004). Thus, the association with the nucleus may reflect the caspase-3 activity. According to the manufacturer, the anti-caspase-2 and -3 antibodies can recognize the pro-enzyme as well as the active form, but we reported in a previous paper (Peluffo *et al.* 2005) that caspase-2 antibody only detected the inactive form of caspase-2 by western blotting under various conditions (data not shown). The discrepancy between the IHC results for caspase-2 and -3 may be related to differences in the specificity of their respective antibodies for the active versus inactive caspase.

The activity for caspase-2 and -3 showed a peak in the old CL at estrus of the natural cycle, associated with the functional regression (i.e., decline in progesterone content and P450scc levels) of the rat CL. The discrepancy between the western blot and the activity results for caspase-3 may be because a processed caspase is not necessarily catalytically active since processing and activation are under the control of different protein factors, such as inhibitor of apoptosis proteins and heat sock proteins. Besides, the activation requires the formation of a tetramer composed of two heterodimers (containing one large and one small subunit). Regarding caspase activity, similar results were obtained in caspase-9 activity, and for this caspase the activity reminds high in the old CL at DII, suggesting an important role of the mitochondria pathway triggering the apoptosis of the rat CL. All these findings suggest that the caspase activation is an early event of the regression of the CL.

CL progesterone levels were measured during the estrous cycle and they had reached peak values by the DII stage in the new CL, in accordance to previous results reported in serum (Smith *et al.* 1975). Accordingly, a significant increase in the P450ssc protein level was found in the same CL stage. As it was expected, the increase in progesterone and P450scc levels is coincident with the functional CL. Previous reports have suggested that progesterone suppresses caspase-3

activity (Svensson *et al.* 2001, Okuda *et al.* 2004), cellular apoptosis (Robker *et al.* 2000), and luteal degeneration (Young & Stouffer 2004) (Duffy & Stouffer 1997), in CL of species with long luteal phases during the ovarian cycle. Nevertheless, we found the peak activity of caspases in the rhesus monkey CL at the mid–late luteal phase of the cycle, when the progesterone levels are still high (Peluffo *et al.* 2005). However, we have to consider the differences between species and time course. In rodent, the estrous cycle lasts only 4–5 days, but the CL lifespan extends for more than one cycle. In our model, further studies are warranted to demonstrate that the high activity levels for caspases found in rat CL have a role in degrading the steroid synthesis machinery in addition to their specific role on apoptosis. In this regard, the association between caspase function and luteolysis is supported by our results obtained in pregnant rats. Caspase-2, -3, -8, and -9 activity levels were eight to tenfold lower in the CL at day 7 of pregnancy in comparison to the CLs from the estrous cycle, showing a decrease in the caspase activities in accordance to further CL development and function. Experiments are in progress in our laboratory to study the precise role of caspases during luteolysis from pregnant rat CL.

In the past few years, evidence suggesting caspase involvement in the CL regression of different species has increased. Rueda *et al.* (1997) reported an increase in caspase-1 mRNA levels in the cow CL coincident with the onset of luteolysis (Rueda *et al.* 1997). He and his colleagues also demonstrated that caspase-3 plays a pivotal role in the structural regression of the rodent CL using caspase-3 null (−/−) mice (Carambula *et al.* 2002). Moreover in rabbits, inhibitors of caspase-1 and -3 homologs suppress an apoptotic phenotype in cultured corpora lutea (Abdo *et al.* 2001). In contrast to the three caspases previously discussed, caspase-8 activity levels remained unchanged in both old and new CLs of rats at estrus and DII. Since the activity levels as well as the protein expression of caspase-8 did not change during the CL lifespan, this caspase may not play a key role during the regression of the rat CL. But as the activity and expression for caspase-8 was detectable in all groups of the CLs, we cannot eliminate a possible alternative function for this protein. Interestingly, caspases could have other functions regulating proliferation, differentiation, or cytokine production (Los *et al.* 1999, Algeciras-Schimnich *et al.* 2002).

TUNEL studies revealed an increase in the number of apoptotic cells in the old CL of rats at estrus and DII in natural cycle, as luteolysis progressed. Interestingly, this increase occurred after the increase in caspase-2, -3, and -9 activities. Nonetheless, luteal cells with advanced signs of apoptosis, as well as the activity for the four caspases studied, were found in all the CLs at estrus or DII. Other reports and our rhesus monkey data (Dharmarajan *et al.* 1994, Shikone *et al.* 1996, Peluffo

et al. 2005) demonstrated nuclear DNA fragmentation in the functional CL.

In summary, dynamic expression of some initiator and effector caspases was observed during the formation and regression of the rat CL. Caspase-2 and -9 as well as the main effector caspase-3 activity increased in the old CL at estrus, during the functional luteolysis. Subsequently, and perhaps as a consequence, the number of apoptotic cells increased, whereas luteal weight decreased, as luteolysis progressed at DII in the old CL. The dynamics of initiator (-2 and -9) and effector (-3) caspase activity suggests an important role for this protease family during the early events of the luteolysis in the rat during the natural estrous cycle.

Acknowledgements

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