



Cleavage of cytoskeletal proteins by caspases during ovarian cell death: evidence that cell-free systems do not always mimic apoptotic events in intact cells

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Abstract

Several lines of evidence support a role for protease activation during apoptosis. Herein, we investigated the involvement of several members of the *CASP* (cysteine aspartic acid-specific protease; CED-3- or ICE-like protease) gene family in fodrin and actin cleavage using mouse ovarian cells and HeLa cells combined with immunoblot analysis. Hormone deprivation-induced apoptosis in granulosa cells of mouse antral follicles incubated for 24 h was attenuated by two specific peptide inhibitors of caspases, zVAD-FMK and zDEVD-FMK (50–500 μ M), confirming that these enzymes are involved in this paradigm of cell death. Proteolysis of actin was not observed in follicles incubated *in vitro* while fodrin was cleaved to the 120 kDa fragment that accompanies apoptosis. Fodrin, but not actin, cleavage was also detected in HeLa cells treated with various apoptotic stimuli. These findings suggest that, in contrast to recent data, proteolysis of cytoplasmic actin may not be a component of the cell death cascade. To confirm and extend these data, total cell proteins collected from mouse ovaries or non-apoptotic HeLa cells were incubated without and with recombinant caspase-1 (ICE), caspase-2 (ICH-1) or caspase-3 (CPP32). Immunoblot analysis revealed that caspase-3, but not caspase-1 nor caspase-2, cleaved fodrin to a 120 kDa fragment, whereas both caspases-1 and -3 (but not caspase-2) cleaved actin. We conclude that *CASP* gene family members participate in granulosa cell apoptosis during ovarian follicular atresia, and that collapse of the granulosa cell cytoskeleton may result from caspase-3-catalyzed fodrin proteolysis. However, the discrepancy in the data obtained using intact cells (actin not cleaved)

versus the cell-free extract assays (actin cleaved) raises concern over previous conclusions drawn related to the role of actin cleavage in apoptosis.

Keywords: apoptosis; caspase; ICE; CPP32; actin; fodrin; proteolysis; granulosa cell; follicle; atresia

Abbreviations: caspase, cysteine aspartic acid-specific protease (*CASP*, designation of the gene); ICE, interleukin-1 β -converting enzyme; zVAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; zDEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; kDa, kilodaltons; ICH-1, ICE/CED-3 homolog-1; CPP32, cysteine protease p32; PARP, poly(ADP-ribose)-polymerase; MW, molecular weight; STSP, staurosporine; C8-CER, C8-ceramide; DTT, dithiothreitol; cCG, equine chorionic gonadotropin (PMSG or pregnant mare's serum gonadotropin); ddATP, dideoxy-ATP; kb, kilobases; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; ANOVA, analysis of variance

Introduction

Physiological cell death is a process that has been identified to occur in many cell types and, when deregulated, may be an underlying mechanism by which tissues reach a pathological or diseased state (Thompson, 1995). Much of the work in this field has been directed towards identifying the components of the cell death machinery that carry out 'the order' to initiate apoptosis. These data suggest that activation of a cascade of intracellular proteases may be integral to this event, the end-result of which leads to the demise of the cell (Yuan *et al*, 1993; Martin and Green, 1995; Kumar and Lavin, 1996; Patel *et al*, 1996). Central to the study of proteases and apoptosis is work that has focused on the identification of vertebrate homologs of the *ced-3* gene product required for programmed cell death in the nematode, *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Yuan *et al*, 1993; Xue *et al*, 1996). To date, at least 11 CED-3 homologs have been identified in vertebrates, and for the sake of clarity these enzymes are now being collectively termed cysteine aspartic acid-specific proteases (caspases; Alnemri *et al*, 1996). All members of the caspase family in vertebrates, including caspase-1 (interleukin-1 β -converting enzyme or ICE), caspase-2 (NEDD-2 or ICH-1) and caspase-3 (CPP32, Yama or apopain) studied herein, share two unique features: a consensus pentapeptide domain (QACXG) containing the cysteine active site, and obligate specificity for cleavage of target proteins at aspartate residues (Martin and Green, 1995; Patel *et al*, 1996; Kumar and Lavin, 1996).

With the recent purification of many of these proteases, several key protein substrates cleaved by members of the *CASP* gene family have been identified. For example, in addition to processing pro-IL1 β and pro-caspase-1 to their active forms, caspase-1 also activates pro-caspase-3. More recently, caspase-1 was reported to cleave actin in a cell-free system, as well as in intact cells during death (Mashima *et al*, 1995; Kayalar *et al*, 1996), possibly as a means to liberate or activate DNase-I (Kayalar *et al*, 1996). In addition to actin, other homeostatic proteins are subject to proteolysis by caspases. These include a second cytoskeletal protein (fodrin; Martin *et al*, 1995, 1996), as well as nuclear-associated proteins such as poly(ADP-ribose) polymerase (PARP), the U1-70 kDa protein component of small nuclear ribonucleoproteins, the catalytic subunit of DNA-dependent protein kinase, and lamins (Martin and Green, 1995; Patel *et al*, 1996; Casciola-Rosen *et al*, 1996).

Despite these advances in our understanding of the role of proteases in apoptosis, there are limitations in the interpretation of the data, particularly those findings related to cleavage of cytoskeletal proteins. For example, caspase-1-deficient mice do not exhibit defects in many instances of apoptosis (glucocorticoid-treated thymus gland, post-weaning mammary gland) (Kuida *et al*, 1995; Li *et al*, 1995), suggesting that either caspase-1-mediated actin cleavage is not required for apoptosis or that an enzyme other than caspase-1 is involved in actin proteolysis. Moreover, data to support the role of actin cleavage during apoptosis are for the most part inconclusive due to the extrapolation of results obtained primarily from cell-free assays to the *in vivo* situation (Mashima *et al*, 1995; Kayalar *et al*, 1996). Data that implicate fodrin proteolysis in apoptosis are more convincing, although several questions remain concerning the identity of the protease(s) responsible for cleavage of this protein (Martin *et al*, 1995, 1996). Thus, we used a combination of intact cell and cell-free systems to elucidate if cleavage of actin or fodrin is observed during apoptosis under diverse conditions, and to identify enzyme(s) capable of cleaving these two cytoskeletal proteins.

Results and Discussion

The intracellular effectors involved in the initiation, progression and completion of apoptosis are believed to be conserved across most species and cell types (Korsmeyer, 1995; Wyllie, 1995). In agreement with this proposal, results from recent studies suggest that apoptotic death of granulosa cells during ovarian follicular atresia is likely dependent upon the actions and interactions of a number of these conserved regulatory factors (reviewed in Tilly, 1996; Tilly and Ratts, 1996; Tilly *et al*, 1997), including members of the *CASP* gene family (Flaws *et al*, 1995a). Consistent with previous findings from other cell types (Patel *et al*, 1996; Cain *et al*, 1996; Jacobson *et al*, 1996), the occurrence of apoptosis in serum-deprived follicles incubated *in vitro* was attenuated by either of two specific peptide inhibitors of caspases (Figure 1). At the highest concentration tested (500 μ M), zVAD-FMK and zDEVD-FMK reduced the levels of low MW DNA labeling, a measure of the extent of apoptosis (see Materials and

Methods) to $39 \pm 5\%$ and $44 \pm 10\%$, respectively, of those levels detected in serum-deprived follicles incubated for 24 h with vehicle alone ($P < 0.05$ for each, $n=5$).

Based on these data that further support a functional role for caspases in granulosa cell death, we next determined if two cytoskeletal elements known to be cleaved by caspases are targeted for destruction during atresia. Immunoblot analysis of actin in total proteins extracted from follicles prior to (0 h; no apoptosis) and after a 48 h serum-free incubation (extensive apoptosis) revealed no evidence of cleavage of the 42 kDa intact form (Figure 2A), contrasting the pattern of actin fragments generated by caspase-1 or caspase-3 attack using a cell-free approach (Figure 3) (Kayalar *et al*, 1996). Similarly, treatment of follicles with staurosporine (STSP), a protein kinase inhibitor that activates apoptosis in essentially all eukaryotic cell systems (Weil *et al*, 1996), further increased apoptosis in serum-starved follicles (data not shown) but did not induce cleavage of actin (Figure 2A).

By comparison, immunoblot analysis of fodrin integrity indicated that follicles induced to undergo atresia *in vitro* by trophic hormone-deprivation exhibited cleavage of the 240 kDa intact form of fodrin to a 120 kDa fragment (Figure 4A). Moreover, STSP treatment caused a further increase in the amount of the 120 kDa fragment generated over that observed in serum-free follicles incubated for 48 h in medium alone (Figure 4A). Importantly, this cytoskeletal element was cleaved in the same protein preparations that clearly displayed a lack of actin proteolysis (Figure 4 *versus* Figure 2, respectively), despite the fact that actin is known to be a substrate for caspase-1 (Kayalar *et al*, 1996).

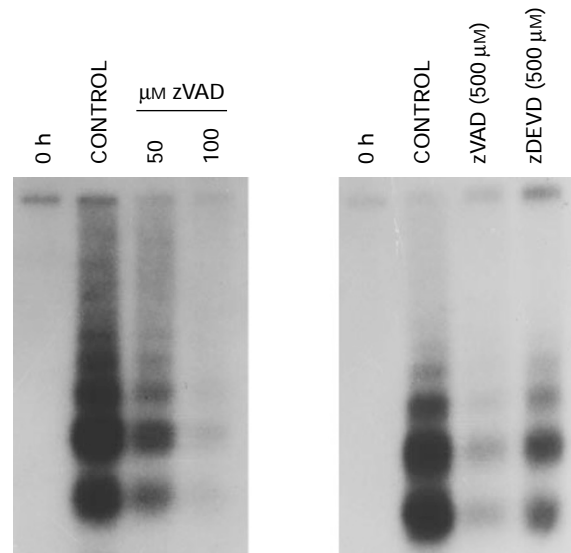


Figure 1 Suppression of apoptosis by specific peptide inhibitors of caspases in mouse ovarian follicles deprived of trophic hormone support *in vitro*. Healthy antral follicles isolated from ovaries of eCG-primed immature mice were snap-frozen immediately (0 h) or were incubated for 24 h without serum in the absence (CONTROL) or presence of zVAD-FMK (50–500 μ M, left and right panels) or zDEVD-FMK (500 μ M, right panel). Genomic DNA was extracted, radiolabeled, resolved by agarose gel electrophoresis, and analyzed by autoradiography for internucleosomal cleavage (DNA 'ladders') characteristic of cells undergoing apoptosis (representative of 5 replicate experiments)

Based on our recent observations that caspase-1, *per se*, is likely not a participant in granulosa cell death during follicular atresia in the rodent ovary (Flaws *et al*, 1995a), these observations were first taken as further evidence that caspase-1 is not involved in this specific paradigm of apoptosis. However, a comparative analysis of actin and

fodrin integrity in HeLa cells under three different experimental conditions that produce extensive apoptosis (UV-irradiation, or treatment with staurosporine or ceramide) yielded a similar set of results (Figures 2B and 4B). In addition, apoptosis in HeLa cells triggered by serum starvation, an approach used in a recent report of caspase-1-mediated actin cleavage in PC12 cells (Kayalar *et al*, 1996), similarly proceeded in the absence of actin proteolysis (data not shown). Since HeLa cells express

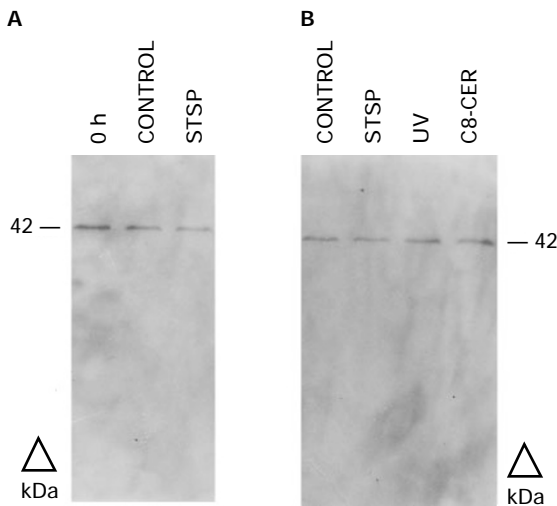


Figure 2 Actin integrity during apoptosis in mouse follicles and HeLa cells. (A) Follicles were frozen (0 h) or incubated without serum for, 48 h without (CONTROL) or with staurosporine (STSP), after which total proteins were extracted and analyzed for actin cleavage by immunoblot analysis. (B) For comparison, actin integrity was also assessed in total proteins prepared from HeLa cells following massive apoptosis induced by STSP, UV-irradiation or a ceramide analog (C8-CER) (representative of 3 independent experiments)

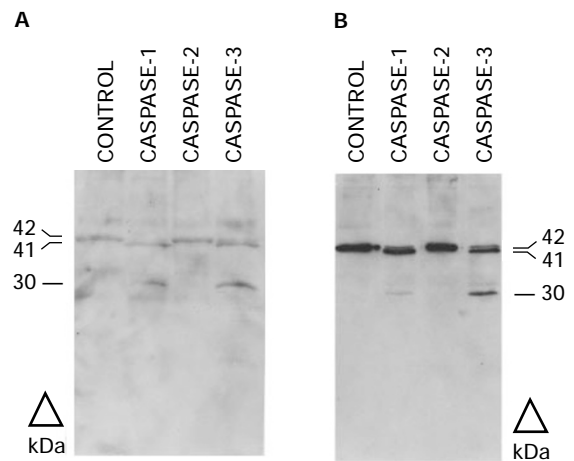


Figure 3 Catalysis of actin cleavage by recombinant caspases in cell-free assays. Total proteins extracted from mouse ovaries (A) or HeLa cells (B) were incubated without (Control) or with caspase-1, caspase-2 or caspase-3, after which immunoblot analysis of actin integrity was conducted (these data are representative of results obtained in at least 3 independent experiments)

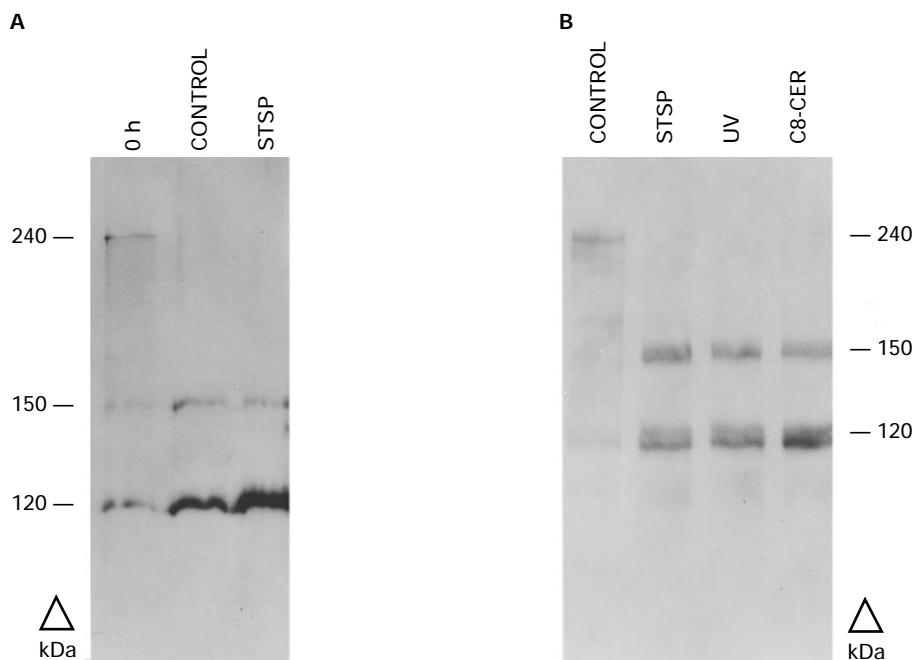


Figure 4 Analysis of fodrin proteolysis during cell death. Using the same protein preparations analyzed in Figure 2, cleavage of fodrin was assessed by immunoblot analysis in mouse follicles (A) or in HeLa cells (B) prior to and following the induction of apoptosis. The culture conditions are as described in the legend to Figure 2, and these blots are representative of data obtained in 3 independent experiments

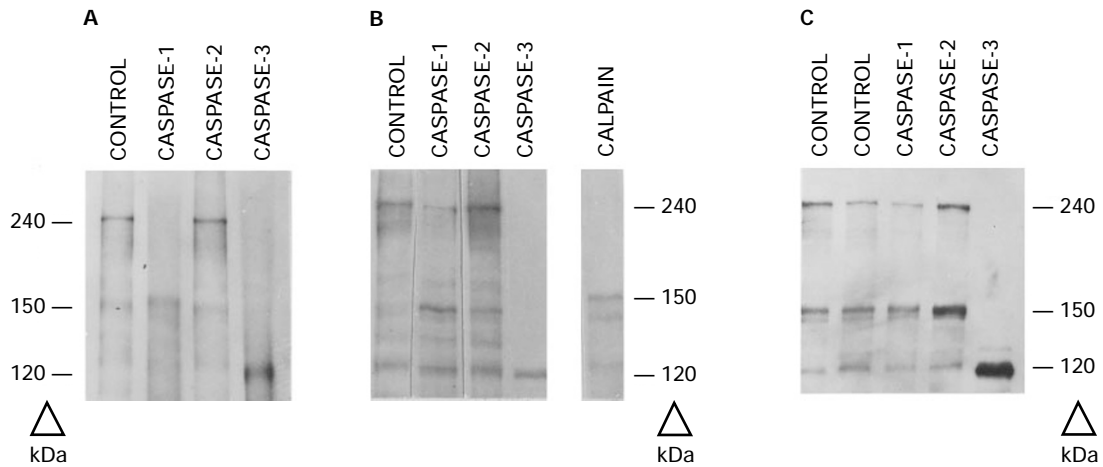


Figure 5 Cleavage of fodrin in cell-free assays by caspases and calpain. Total cell proteins prepared from mouse ovaries (A) or HeLa cells (B) as described in legend to Figure 3, as well as from rat ovarian granulosa cells (C), were incubated without (CONTROL) or with recombinant caspases, and fodrin proteolysis was assessed by immunoblot analysis. Based on recent data implicating calpain as one possible mediator of fodrin cleavage during cell death (Martin *et al*, 1995), the pattern of fodrin proteolysis obtained with recombinant caspases was also compared to that generated by calpain in HeLa cell extracts (B). The blots shown are representative of data obtained in at least 3 independent experiments

abundant levels of caspase-1 and related enzymes (Wang *et al*, 1994; Friedlander *et al*, 1996), these data argue that cleavage of actin by caspase-1 (or other proteases) is not an event required for, or even correlated with, apoptosis.

To confirm the specificity and sensitivity of this approach for monitoring proteolysis during cell death, we next used recombinant or purified enzymes in a cell-free assay, as described in recent investigations (Bump *et al*, 1995; Kamens *et al*, 1995; Kayalar *et al*, 1996). Total protein extracts prepared from either mouse ovaries or HeLa cells were incubated with recombinant caspases or purified calpain and immunoblots were then performed. Analysis of fodrin indicated that caspase-3, caspase-1 and calpain, but not caspase-2, catalyzed cleavage of this protein (Figure 5). Calpain was originally proposed as the enzyme responsible for fodrin proteolysis during cell death (Martin *et al*, 1995). Although we could demonstrate calpain-mediated fodrin cleavage in the cell-free assay, it is important to point out that consistent with previous data (Saido *et al*, 1993) the fragment produced by the actions of calpain was 150 kDa (Figure 5B). During cell death, however, a 120 kDa fragment of fodrin is generated (Figure 4) (Martin *et al*, 1995, 1996). This cleavage product generally predominates on immunoblots of proteins extracted from apoptotic cells (Figure 4) (Martin *et al*, 1995, 1996), and is also the primary fragment produced specifically by the actions of caspase-3 (Figure 5). Therefore, as originally proposed (Saido *et al*, 1993), it may be that calpain is involved in fodrin turnover in the cell, albeit its function in this regard related to cell death remains an open question. However, these data do not rule out the involvement of calpain in other aspects of apoptosis execution as described previously (Squier *et al*, 1994). Of further note, caspase-1-mediated fodrin proteolysis similarly produced only the 150 kDa fragment (Figure 5). Therefore, based on our data and recent observations that fodrin proteolysis is blocked by a specific peptide inhibitor of

caspase-3 (DEVD; Martin *et al*, 1996), it may be that caspase-3 is a principal cytoplasmic protease responsible for complete fodrin breakdown during apoptosis.

Lastly, generation of the 41–42 kDa actin doublet from the intact 42 kDa cytoplasmic protein was produced by the action of either caspase-1 or caspase-3 in our cell-free assays (Figure 3). In the study by Kayalar and colleagues (1996), one immunoblot was presented that showed a minor actin cleavage product of 30 kDa in PC12 cells induced to undergo apoptosis by serum starvation; however, the 41–42 kDa actin doublet produced by the actions of caspase-1 or caspase-3 in cell-free assays was not apparent. Nevertheless, it was proposed that cytoplasmic disruption and DNase-I activation during apoptosis result from caspase-1-mediated actin cleavage (Kayalar *et al*, 1996). In our experiments, extensive apoptosis induced in either follicles or HeLa cells occurred without cleavage of actin (Figure 2), despite the induction of caspase activity as evidenced by fodrin proteolysis in the same protein preparations (Figure 4). Although proteolysis of actin was not detected during apoptosis induced under diverse conditions in two different cell lineages, actin cleavage was produced with recombinant caspase-1 or caspase-3 in the cell-free assays (Figure 3).

Conclusions

The data presented extend earlier observations (Flaws *et al*, 1995a) by supporting a role for caspases and fodrin proteolysis in the cascade of events leading to granulosa cell apoptosis during ovarian follicular atresia. More importantly, however, these data reinforce the fact that observations made using cell-free approaches (actin cleavage by caspases) may not always be directly applicable to apoptosis in intact cells (actin not cleaved despite the fact caspases are active as evidenced by the inhibitory effects of zVAD and DEVD on apoptosis induction as well as by the

occurrence of fodrin proteolysis). Preliminary findings demonstrate that in the case of PARP and DNA topoisomerase-I cleavage, two additional substrates for caspases, proteolysis of these proteins occurs with a different pattern of fragments produced, depending on if apoptotic or pathologic (necrotic) cell death ensued (Casiano and Tan, 1996). This would imply that different cell death mechanisms likely activate separate cohorts of proteases. Given that the kinetics of protein cleavage during apoptosis are different from the kinetics observed during primary necrosis, the specific cleavage products of the substrates described herein following incubation with recombinant enzymes should be carefully compared to those identified under particular paradigms of cell death. As exemplified by the discrepancy in results obtained with actin cleavage in cell-free *versus* intact cell systems presented herein, caution should be exercised in the interpretation of such findings from previous and future studies of apoptosis regulation.

Materials and Methods

Reagents

Human recombinant caspase-1 (ICE), caspase-2 (ICH-1) and caspase-3 (CPP32) were prepared as N-His-affinity-tagged proteins in *Escherichia coli* and activated prior to each experiment by dithiothreitol (DTT) treatment (Bump *et al*, 1995; Kamens *et al*, 1995). Cell-permeable fluoromethylketon (FMK) derivatives of the peptide inhibitors of caspases, zVAD and zDEVD (Thornberry *et al*, 1992; Wilson *et al*, 1994; Nicholson *et al*, 1995; Patel *et al*, 1996), were obtained from Enzyme Systems Products (Dublin, CA, USA). Equine CG (PMSG; 2180 IU/mg) and calpain I (80 kDa subunit purified from porcine erythrocytes; 120 U/mg) were obtained from Calbiochem (La Jolla, CA, USA), and cell culture medium and supplements were from Gibco-BRL (Gaithersburg, MD, USA). Anti-fodrin monoclonal antibody #1622 was obtained from Chemicon (Temecula, CA, USA), and anti-actin monoclonal antibody #C4 was obtained from Boehringer-Mannheim (Indianapolis, IN, USA).

Animals and tissues

Immature female B6/C3 F1 mice, obtained from Charles-River (Wilmington, MA, USA) at 21 days of age, were given a single s.c. injection of 5 IU of eCG to promote growth of a cohort of healthy antral follicles. Mice were euthanized 46 h later for collection of ovaries and dissection of follicles, as described (Tilly *et al*, 1992, 1995). In some experiments follicles were incubated for 24 h in the absence or presence zVAD-FMK or zDEVD-FMK, and DNA integrity was then assessed (apoptosis). In other experiments follicles were incubated for 48 h without or with 1 mM staurosporine (STSP; Sigma; St. Louis, MO, USA), after which follicles were processed for immunoblot analysis of fodrin and actin integrity in total protein extracts. All parameters for the follicle incubations were as detailed previously (Tilly *et al*, 1992, 1995). Recent studies have shown that rodent follicles incubated *in vitro* without trophic hormone support undergo a process that resembles atresia *in vivo* by all criteria examined and that the occurrence of apoptosis (defined by both morphological and biochemical criteria) is almost entirely restricted to granulosa cells (Tilly *et al*, 1992, 1995, 1996; Flaws *et al*, 1995a,b).

For additional comparisons to data obtained with mouse or human (HeLa) cells, in one set of experiments female Sprague-Dawley rats

(Charles-River) were injected with 10 IU of eCG at 25 days of age and euthanized 46 h later for collection of the ovaries (Tilly *et al*, 1992, 1995). Granulosa cells were harvested from the healthy cohort of gonadotropin-stimulated preovulatory follicles by selective needle puncture (Tilly *et al*, 1992, 1995). Total protein extracts were then prepared and used for cell-free proteolysis assays as described below.

All experimental protocols involving animals were reviewed and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee, and conformed with procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Cell culture

HeLa cells (ATCC CCL-2, Batch F-12477; American Type Culture Collection, Rockville, MD, USA) were grown and subcultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. When approximately 80% confluence was reached, cells were either maintained (controls) or were induced to undergo apoptosis by UV-irradiation (120 mJ, 25 s), by treatment with STSP (0.5 μ M) or C8-ceramide (0.1 mM; Calbiochem), or by serum-starvation. Cells were collected 16–18 h after experimental manipulation involving UV, STSP or C8-CER, or 120 h after serum starvation, and processed for immunoblot analysis of fodrin and actin integrity in total protein extracts. By morphology (condensation, pyknosis, detachment), greater than 85% of the HeLa cells were apoptotic after each experimental treatment (data not shown).

Measurement of DNA integrity (oligonucleosome formation)

Genomic DNA was extracted from follicles and 3'-end-labeled with [α - 32 P]-ddATP (Amersham; Arlington Heights, IL, USA) using terminal transferase (Boehringer-Mannheim). Radiolabeled samples were resolved through 2% agarose gels, and the extent of apoptosis was assessed by autoradiography and β -counting of low MW (< 10 kb) DNA fragments, as described (Tilly and Hsueh, 1993; Tilly, 1994).

Cell-free proteolysis assays and immunoblot analysis

Total protein was extracted from mouse or rat ovarian cells, or from HeLa cells, by homogenization in caspase activity buffer (25 mM HEPES, 10% glycerol, 10 mM DTT). Samples were then boiled for 10 min to destroy endogenous protease activity, sonicated for 30 s to shear DNA, and centrifuged (10 000 \times g, 10 min, 4°C). Supernatants were collected for protein quantitation (Pierce MicroBCA; Rockford, IL, USA) and proteolytic digestion assays. Proteins (200 μ g) from each cellular preparation were incubated with 1 μ g of activated recombinant caspase (caspase-1, -2 or -3) or purified calpain for 2 h at 37°C, and then resolved by SDS-PAGE through 8% (fodrin) or 15% (actin) gels. Resolved proteins were electrophoretically transferred to pure nitrocellulose membranes (Schleicher and Schuell; Keene, NH, USA) and analyzed by immunoblot analysis for fodrin or actin cleavage, essentially as described (Flaws *et al*, 1995a), using the enhanced chemiluminescence system (Amersham).

Data analysis and presentation

Representative autoradiograms or immunoblots are presented for qualitative analysis of data obtained in at least three independent experiments. Combined data for oligonucleosome formation across replicate experiments were analyzed by ANOVA and Scheffe's F-test, with significance assigned at $P < 0.05$.

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