Expression and activity of Apaf1 and caspase-9 in granulosa cells during follicular atresia in pig ovaries

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Apoptosis in granulosa cells plays a crucial role in ovarian follicular atresia, but the intracellular regulating mechanism, especially the mitochondrion-dependent apoptosis signalling pathway, is still largely unknown. This study examined whether the mitochondrial pathway is associated with granulosa cell apoptosis during atresia in pig ovaries. Both mRNAs of caspase-9 and apoptotic protease-activating factor 1 (Apaf1), which are major signal transducing components in the mitochondrial pathway, were detected in granulosa cells in healthy, early atretic and progressed atretic follicles by RT–PCR. No changes in the expression of Apaf1 mRNA were seen during follicular atresia, but the expression of caspase-9 mRNA increased during atresia. Apaf1 protein was steadily detected in granulosa cells prepared from healthy, early atretic and progressed atretic follicles by western blot analysis, but high expression of the precursor of caspase-9 (procaspase-9) was detected only in granulosa cells of healthy follicles. Decreased procaspase-9 protein was demonstrated during follicular atresia. Proteolytic activity of caspase-9 increased during atresia, in agreement with the diminution of procaspase-9 protein. Intensive expression of caspase-9 mRNA was demonstrated in the granulosa cells of early atretic and progressed atretic follicles but not in those of healthy follicles. These results indicate that the mitochondrial signalling pathway, which is mediated by Apaf1 and caspase-9, plays a crucial role in determining the fate of granulosa cells during atresia in pig ovaries.

Introduction
In mammalian ovaries, more than 99% of the follicles undergo atresia at various stages of follicle development (Tilly, 1996, 1998; Kaipia and Hsueh, 1997). Manabe et al. (1996a, 1997, 1998) indicate that the degeneration of atretic follicles in mammalian ovaries can be explained, at least in part, by apoptotic cell death of granulosa cells. In the early stage of atresia in pig ovaries, apoptosis demonstrated histochemically and ultrastructurally was seen in scattered granulosa cells located on the inner surface of the follicular wall, but not in cumulus cells, oocytes or the cells of internal or external theca layers (Manabe et al., 1996a; Sugimoto et al., 1998). However, the apoptotic signal transducing pathway system that mediates apoptosis in granulosa cells of pig ovaries is not known.

The process of apoptosis is triggered by diverse signals (Tilly, 1996) and various stimuli of apoptosis lead to the activation of intracellular caspase cascades, in which at least 14 caspases have been identified to date (Grutter, 2000). Some of the caspase cascades mediate apoptotic cell death in mammalian ovaries (Khan et al., 2000; Johnson and Bridgham, 2002). There are two major apoptosis signalling pathways: cell-death receptor-dependent and mitochondrion-dependent (Nagata, 1997; Ashkenazi and Dixit, 1998; Green and Reed, 1998). The receptor-dependent pathway is triggered by members of the death receptor superfamily, Fas/APO1/CD95, tumour necrosis factor (TNF) receptor-1 and -2, and TNF-related apoptosis-inducing ligand (TRAIL) receptor-4 and -5. In the mitochondrion-dependent pathway, various cellular stresses induce cytochrome c release from the mitochondria; and cytochrome c binds to apoptotic protease-activating factor 1 (Apaf1) (Zou et al., 1997; Robles et al., 1999), which in turn self-associates and binds with procaspase-9, a zymogen of caspase-9. Procaspase-9 protein, although limited, has proteolytic activity without being cleaved (Stennicke et al., 1999). Generally, the complex molecule (cytochrome C–Apaf1–procaspase-9), called apoptosome, cleaves procaspase-9 protein and makes it active, and then the cleaved or activated caspase-9 activates downstream caspases, such as caspase-3 (Grutter, 2000; Wang, 2001). In granulosa cells of healthy follicles, the inactive form of caspase-3 (procaspase-3) was demonstrated, but procaspase-3 protein was cleaved and activated in granulosa cells prepared from atretic follicles (Izawa et al., 1998; Manabe et al., 1998). It is not yet

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understood which apoptosis signalling pathway plays a critical role in determining granulosa cell apoptosis during atresia in pig ovaries.

In the present study, attempts were made to determine the role of the mitochondrion-dependent signalling pathway in granulosa cell apoptosis under physiological conditions. The changes in expression of Apaf1 and procaspase-9 proteins, and Apaf1 and caspase-9 mRNAs were examined by western blot analysis and RT–PCR, respectively, and the changes in the activity of caspase-9 were assessed biochemically. In addition, the changes in the localization of caspase-9 mRNA were investigated by in situ hybridization.

Materials and Methods

Preparation of granulosa cells

The ovaries were obtained from mature sows of >120 kg body weight from a local abattoir. Individual antral follicles, approximately 3 mm in diameter, were dissected from the ovaries under a surgical dissecting microscope (SZ40, Olympus, Tokyo). Each follicle was classified as morphologically healthy or atretic and further subdivided into early and progressed atretic follicles (approximately 15 follicles per ovary were used). Follicular fluid from each follicle was collected using a 1 ml syringe, separated by centrifugation at 3000 g for 10 min at 4°C, and frozen and stored at −80°C. After biochemical analyses were performed, oestriadiol and progesterone concentrations were measured retrospectively using [125I]-radioimmunoassay kits (Bio-Mérieux, Marcy-l’Etoile) to confirm the classification of the follicles. Follicles with a progesterone:oestradiol ratio of less than 15 were classified as healthy (Guthrie et al., 2002; Nakayama et al., 2003), for RT–PCR analysis and cDNA sequencing for 10 min at 4°C.

Western blot analysis

As previously reported (Nishihara et al., 2000; Nakayama et al., 2003), for western blot analysis the protein fraction (50 μg per lane) prepared from each sample was separated by 10–20% gradient SDS-PAGE (Wako) and then transferred on to nitrocellulose membranes (Hybond-C, Amersham Pharmacia). The membranes were stained with 0.2% (w/v) Ponceau S solution (Serva Electrophoresis, Heidelberg) and then immersed in blocking solution (0.1 mol Tris–HCl 1–1, pH 7.6, 5% (w/v) skimmed milk, 0.05 mol NaCl 1–1, 0.1% (v/v) Tween 20; Sigma) for 30 min, and incubated with rabbit polyclonal anti-Apaf1 antibody (1:2000 dilution with blocking solution; Chemicon International, Temecula, CA) or rabbit polyclonal anti-procaspase-9 (1:2000 dilution with blocking solution; Pharmingen, San Diego, CA) for 18 h at 4°C. After washing, the membranes were assessed.
were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson Immuno-Research Laboratories, West Grove, PA) for 1 h at 25°C and then chemiluminescence was visualized using an ECL system (Amersham Pharmacia) according to the manufacturer’s protocol. The chemiluminescence was recorded with a digital fluorescence recorder, and then each protein expression level, the chemiluminescence intensity of each protein band, was quantified using ImageGauge on a Macintosh computer.

Enzyme activity assay

The proteolytic activity of caspase-9 was determined using the caspase-9 colorimetric protease assay kit (MCH-6; Chemicon) according to the manufacturer’s protocol. Briefly, fresh granulosa cells prepared from healthy, early atretic and progressed atretic follicles were washed in PBS and then lysed in lysis solution (50 mmol Tris–HCl l$^{-1}$, pH 7.4, 150 mmol NaCl l$^{-1}$, 20 mmol EDTA l$^{-1}$, 50 mmol NaF l$^{-1}$, 0.5% (v/v) Nonidet P-40, 0.1 mmol Na$_2$VO$_4$ l$^{-1}$, 20 mg leupeptin ml$^{-1}$, 20 mg aprotinin ml$^{-1}$, 1 mmol dithiothreitol l$^{-1}$ and 1 mmol phenylmethylsulphonyl fluoride l$^{-1}$). After centrifugation at 10,000 g for 5 min, each supernatant was collected and protein concentration in the supernatant was measured by the Bradford assay kit (Bio-Rad Laboratories, Melville, NY). The supernatant was incubated with LEHD-p-nitroaniline (LEHD-pNA; a colorimetric peptidyl substrate for caspase-9) for 2 h at 37°C. Free pNA was measured using a microplate reader (Model 450; Bio-Rad) at a wavelength of 405 nm to assess the proteolytic activity of caspase-9.

Apoptotic cell detection and in situ hybridization

Apoptotic cells in pig ovarian sections were detected immunohistochemically according to the method described by Watanabe et al. (1999). Briefly, frozen serial sections 5 µm thick, mounted on glass slides pre-coated with 3-aminopropyltriethoxysilane (Sigma) were fixed with 10% phosphate buffered formalin. After washing with PBS, the sections were treated with 0.3% (v/v) H$_2$O$_2$ methanol for 30 min and washed with PBS containing 0.02% (v/v) Tween 20 (PBST; Sigma). The sections were then incubated with polyclonal rabbit anti-single strand DNA (ssDNA) antibody (1:100 diluted with PBS; Dako, Glostrup) for 18 h at 4°C, washed with PBST and incubated with Envision solution for rabbit antibody (Dako) for 60 min. After washing with PBST, the sections were reacted with 0.05% (w/v) 3,3’-diaminobenzidine (Wako) and 0.002% (v/v) H$_2$O$_2$ in 50 mmol Tris–HCl l$^{-1}$, pH 7.2, for 1 min at 25°C, washed with distilled water, counterstained with haematoxylin, mounted with Entellan (Merck, Darmstadt) and examined using light microscopy.

The localization of caspase-9 mRNA by in situ hybridization was visualized by synthesizing digoxigenin (DIG)-labelled antisense and sense cRNA probes for caspase-9 mRNA using a Lig’n Scribe kit (Ambion, Austin, TX) and DIG-RNA-labelling kit (Roche Diagnostics, Rotkreuz) according to the manufacturer’s protocols. Frozen ovarian sections were fixed with 4% (v/v) paraformaldehyde (PFA; Wako) in PBS for 8 min, washed with PBS and treated with 0.2 mol HCl l$^{-1}$ for 10 min at room temperature (air-conditioned, 22–25°C). After washing with PBS, the sections were digested with proteinase K (1 µg ml$^{-1}$; Sigma) in PBS at 37°C for 10 min, post-fixed with 4% PFA in PBS, immersed with 2 µg glycine ml$^{-1}$ in PBS for 3 min, and washed thoroughly with PBS. The sections were prehybridized with 10 mmol Tris–HCl l$^{-1}$, pH 7.4, containing 600 mmol NaCl l$^{-1}$, 1 x Denhardt’s solution, 50% (v/v) deionized formamide for 1 h at 25°C, and hybridized with an antisense–DIG–caspase-9 cRNA probe for 18 h at 45°C. Each probe (1 µg ml$^{-1}$) was diluted in 10 mmol Tris–HCl l$^{-1}$, pH 7.4, 1 mmol EDTA l$^{-1}$, 600 mmol NaCl l$^{-1}$, 1 x Denhardt’s solution, 10 mg yeast tRNA ml$^{-1}$, 10 mg salmon testicular DNA ml$^{-1}$, 5% (v/v) dextran sulphate and 50% (v/v) deionized formamide. The sections were washed with 2 × standard saline citrate (SSC) for 2 h, 0.5 × SSC for 2 h and 0.2 × SSC for 1 h at 45°C, and then equilibrated with 100 mmol Tris–HCl l$^{-1}$, pH 7.5, containing 150 mmol NaCl l$^{-1}$ (THS). Sections were then treated with blocking solution (Roche) for 1 h at room temperature, incubated with alkaline phosphatase (AP)-conjugated sheep anti-DIG antibody (1:500 diluted with blocking solution; Roche) for 18 h at 4°C, washed with THS, and rinsed with 100 mmol Tris–HCl l$^{-1}$, pH 9.5, 100 mmol NaCl l$^{-1}$ and 100 mmol MgCl$_2$ l$^{-1}$ (THSM). They were immersed with THSM containing 0.4 mmol nitroblue tetrazolium chloride l$^{-1}$ (Sigma), 0.4 mmol 5-bromo-4-chloro-3-indolylphosphosphate4-toluidine salt l$^{-1}$ (Sigma) and 1 mmol levamisole l$^{-1}$ (Sigma), and then incubated for 90 min at 25°C. The sections were immersed with PBS, mounted with Histofine (Nichirei, Tokyo) and examined under a light microscope. Dimethyl pyrocarbonate (Sigma)-treated water was used throughout the staining processes. As negative controls, serial sections were hybridized with a DIG-labelled sense cRNA probe or without any probes, or incubated without anti-DIG antibody. Any controls yielded completely negative results.

Statistical analysis

All experiments including follicle isolation and granulosa cell preparation were repeated three times with separate groups (nine sows per group) for independent observation. Homogeneity of variance was assessed using StatView 4.5 (Abacus Concepts, Berkely, CA) on a Macintosh computer before ANOVA processing. ANOVA with Fisher’s least significant differences test for biochemical data and Wilcoxon’s signed-rank tests
Fig. 1. RT–PCR products, expression and chemiluminescence western blot analysis for caspase-9, apoptotic protease-activating factor 1 (Apaf1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (a) RT–PCR products for caspase-9, Apaf1 and GAPDH mRNAs. (b) Caspase-9 and Apaf1 mRNA expression (caspase-9 mRNA:GAPDH mRNA and Apaf1 mRNA:GAPDH mRNA ratios, respectively) in granulosa cells of healthy (H), early atretic (EA) and progressed atretic (PA) pig follicles. (c) Chemiluminescence western blot analysis for procaspase-9 (the precursor of caspase-9) and Apaf1 proteins in granulosa cells from healthy, early atretic and progressed atretic pig follicles. (d) Procaspase-9 and Apaf1 proteins in granulosa cells from healthy, early atretic and progressed atretic follicles were quantified by western blot image analysis and shown as chemiluminescence intensity. All data in (b) and (d) are shown as means ± SEM. *P < 0.05 and ***P < 0.001 versus each healthy sample.

Results

Expression of Apaf1 and caspase-9 mRNAs in granulosa cells

The RT–PCR products detected in the present study were confirmed to be pig Apaf1 and caspase-9 by the cDNA sequence determination. The cDNA sequence of the corresponding domain of Apaf1 was 5′-GATCG GAATG GAAAG TCGTT TCGTT ATTAT TTACA TGATC TTCAA GTAGA TTITT CTACA GAGAA GAATT GCAGC CAGCT TCCAG AATCA CATAA GAAGA TTACAA ACTTT CTGGC CTATC ACGTG GCCAG TGCCA AGATG CACAA GGAAC TTGTG GCTTT ATATG TTCCC TGCGA TTGGA TTAAA GCAA AACCAG AACAT GTAGG CCCTG CTATC CTGAT TCGTT AGATG TGGGA ATACA GACAT ATACT AGATG AAAAA GATTG TGCGA TGAGA TTCCC AGGAG TTTTT ATCTT TAAAT GAGACA CTTTC TTGGA CGACA GCGAT TCCTC AATAT TGTCG AACTG GGCTG CTGTG AGCCG GAAAC TTCAG AAGTT TATCA GCAAG CTAGA CTAAG CTGCA GGCCA AGCAG GAGGT CGATA ATGGA ATG-3′ (498 bp). The similarity between pig and human Apaf1 (GenBank Accession No. AJ243048) was 99.6%. The cDNA sequence of the corresponding domain of caspase-9 was 5′-GGCTG TCTAC GGCAC AGATG GATGC CCTGT GTCCC AAGAT TGTGA ACATC TTCAA GTGGA CCAGC TGCCC CAGCC TTGGG GGAGA GCCCA AGATG GAGGT CGATA ATGGA ATG-3′ (498 bp). The similarity between pig and human Apaf1 (GenBank Accession No. AJ243048) was 99.6%. The cDNA sequence of the corresponding domain of caspase-9 was 5′-GGCTG TCTAC GGCAC AGATG GATGC CCTGT GTCCC AAGAT TGTGA ACATC TTCAA GTGGA CCAGC TGCCC CAGCC TTGGG GGAGA GCCCA AGATG GAGGT CGATA ATGGA ATG-3′ (498 bp). The similarity between pig and human Apaf1 (GenBank Accession No. AJ243048) was 99.6%. The cDNA sequence of the corresponding domain of caspase-9 was 5′-GGCTG TCTAC GGCAC AGATG GATGC CCTGT GTCCC AAGAT TGTGA ACATC TTCAA GTGGA CCAGC TGCCC CAGCC TTGGG GGAGA GCCCA AGATG GAGGT CGATA ATGGA ATG-3′ (498 bp).
Apaf1 and caspase-9 in pig granulosa cells

Changes in expression of Apaf1 and procaspase-9 proteins in granulosa cells

No significant differences in Apaf1 protein expression among healthy, early atretic and progressed atretic follicles were shown in all stages of follicles (Fig. 1c,d). Strong immunoreaction for procaspase-9 protein (precursor protein of caspase-9) was demonstrated in the granulosa cells of healthy follicles. Decreased expression of procaspase-9 protein was seen in granulosa cells from early atretic follicles, and no expression of procaspase-9 protein was detected in those from progressed atretic follicles (Fig. 1c). Changes in the chemiluminescence intensity quantified using an automatic image analyser showed that procaspase-9 mRNA expression decreased and finally disappeared during follicular atresia (P < 0.001; Fig. 1d).

Changes in activity of caspase-9 in granulosa cells

Higher proteolytic activities of caspase-9 in isolated granulosa cells of early atretic and progressed atretic follicles than in those of healthy follicles were determined (P < 0.001), indicating the activation of caspase-9 during follicular atresia (Fig. 2).

Localization of apoptotic cells and caspase-9 mRNA in follicles

No ssDNA-positive apoptotic cell was observed in granulosa cells, internal thecal cells or external thecal cells of healthy follicles (Fig. 3a). Similar to previous studies (Manabe et al., 1996a, 1998; Nakayama et al., 2000, 2003), apoptosis occurred in granulosa cells located on the inner surface of the follicular wall in the follicles at the early stage of atresia (Fig. 3d), and positive reaction was seen in most granulosa cells of progressed atretic follicles (Fig. 3g).

In healthy follicles, trace or weak reaction for caspase-9 mRNA was seen in granulosa cells and negative staining was observed in internal thecal cells or external thecal cells (Fig. 3b). Strong staining for caspase-9 mRNA was demonstrated in granulosa cells of both early atretic and progressed atretic follicles (Fig. 3e,h, respectively), indicating the increase in caspase-9 mRNA expression in granulosa cells during follicular atresia. Negative staining for caspase-9 mRNA was seen in internal thecal cells or external thecal cells of early atretic follicles (Fig. 3e), and trace or weak reaction was observed in internal thecal cells of progressed atretic follicles (Fig. 3h). No positive reaction was seen in the sense-probe stainings used as negative controls (Fig. 3c,f,i).

Discussion

It has not been shown clearly which molecule(s) acts as an initial trigger for granulosa cell apoptosis during follicular atresia in mammalian ovaries (Tilly, 1996, 1998; Kaipia and Hsueh, 1997). Balance of survival and death factors is essential for the determination of growth, proliferation, differentiation and cell death in granulosa cells (Markstrom et al., 2002). Although the death receptor-dependent signalling pathway has been investigated thoroughly (Nagata, 1997; Ashkenazi and Dixit, 1998), there is insufficient information on the role of intracellular signalling molecules involving the mitochondrion-dependent signalling pathway in follicles of mammalian ovaries (Kaipia and Hsueh, 1997; Hsu and Hsueh, 2000). Until now, no report concerning the relationship between the Apaf1 and caspase-9 system...
Fig. 3. Ovarian sections from (a,b and c) healthy, (d,e and f) early atretic and (g,h and i) progressed atretic pig follicles. Apoptotic cells were demonstrated immunohistochemically using anti-single strand DNA (ssDNA) antibody (a,d and g). Representative sections were subjected to in situ hybridization with antisense cRNA probe for caspase-9 mRNA (b,e and h), and sense probe as negative control (c,f and i). No ssDNA positive cell was observed in healthy follicles (a). ssDNA positive apoptotic cells were seen in granulosa cells located on the inner surface of the follicular wall in the follicles at the early stage of atresia (d), and positive reaction was seen in most granulosa cells of progressed atretic follicles (g). Trace or weak reaction for caspase-9 mRNA was seen in granulosa cells of healthy follicles (b). Intense staining was seen in granulosa cells of both early atretic and progressed atretic follicles (e and h, respectively). No positive reaction was seen in sense-probe stainings (c,f and i). g: granulosa cell layer; ti: theca interna layer. Scale bars represent 50 μm.

and granulosa cell apoptosis in a physiological situation has been published. The present study investigated whether the mitochondrion-dependent signalling pathway is involved in granulosa cell apoptosis during atresia in pig ovaries. In the mitochondrial signalling pathway, it is generally believed that when the cells receive a stressful stimulus which leads to mitochondrial damage, cytochrome c is released from the mitochondria (Green and Reed, 1998; Wang, 2001) and binds with Apaf1 (Zou et al., 1997; Robles et al., 1999), and then the Apaf1–cytochrome c complex binds with procaspase-9 (Ceconi et al., 1998; Qin et al., 1999). The Apaf1–cytochrome c–procaspase-9 complex plays a key role in mitochondrion-dependent apoptosis (Ceconi, 1999; Grutter, 2000; Wang, 2001). The present study confirmed the constant expression of Apaf1 mRNA and Apaf1 protein in granulosa cells of healthy, early atretic and progressed atretic follicles, indicating that Apaf1 is expressed regardless of whether cells undergo apoptosis. However, proteolytic activity of caspase-9 in granulosa cells increased during follicular atresia, and inactive zymogen, procaspase-9 protein, decreased in granulosa cells. These data indicate that during follicular atresia, cytochrome c released from the mitochondria binds with Apaf1 in granulosa cells, cytochrome c–Apaf1 complex binds with procaspase-9, which is cleaved and processed to its active form (caspase-9), and this leads to the processing and activation of procaspase-3, a downstream executor of apoptosis (Grutter, 2000). Thus, the authors consider that the mitochondrion-dependent signalling
pathway is involved in granulosa cell apoptosis during follicular atresia in pig ovaries. The mitochondrial-dependent signalling pathway may not act as an initial trigger for granulosa cell apoptosis, but may contribute to amplification and propagation of apoptotic cell death in granulosa layers, and consequently rapidly remove atretic follicles.

The detailed mechanism underlying cytochrome c release and its control by regulating factors, the Bcl2 family proteins, in ovarian tissues remains unclear (Vaux et al., 1988; Green and Reed, 1998; Cecconi, 1999; Hsu and Hsueh, 2000; Wang, 2001). In the mitochondrial signalling pathway, Bcl2 family proteins play bifacial roles, as apoptosis-inducing or apoptosis-inhibiting factors (Cecconi, 1999). Diva/Boo, a novel anti-apoptotic Bcl2 family protein, is expressed restrictively in the ovary and epididymis of adult rodents and binds directly to Apaf1 (Inohara et al., 1998; Song et al., 1999). The binding of Diva/Boo with Apaf1 may induce conformation changes in the Apaf1 protein and thus inhibit the recruitment and processing of procaspase-9 protein. Regulator molecules, like Diva/Boo, functioning on the upstream of mitochondrion-dependent signalling in granulosa cells of pig ovaries, need to be determined in the future.

Recently, perplexing findings on the mitochondrion-dependent signalling pathway have been reported in which cytochrome c release from the mitochondria has been observed in cells undergoing apoptosis induced by a cell-death ligand–receptor (FasL–Fas and TNFα–TNF–receptor-1) (Hsu and Hsueh, 2000). For instance, Bid, which is a protein known to interact with Bcl2 protein and other Bcl2 family proteins, acts as a specific proximal substrate of caspase-8 (a major caspase in the death receptor-dependent signalling pathway) in the cells with apoptosis induced by the FasL–Fas system (Thornberry and Lazebnik, 1998; Grutter, 2000). Activated caspase-8 truncates the full-length Bid protein, and then the truncated or activated Bid induces the release of cytochrome c from the mitochondria to cytosol (Luo et al., 1998). Consequently, cytochrome c–Apaf1–caspase-9 complex-dependent apoptosis is induced. There have been no reports of whether there is such an intracellular cross-talk molecule in granulosa cells. In mammalian oocytes, the death-receptor-dependent signalling pathway, Fasl–FasL (Sakamaki et al., 1997; Porter et al., 2001), TRAIL–TRAIL receptors (Wada et al., 2002), TNFα–TNF–receptor-2 (which acts as a survival factor in granulosa cells during follicular growth in pig ovaries) (Nakayama et al., 2003), and other cell-death ligand–receptor systems (Manabe et al., 1998, 2000) may contribute to the follicular selection process. As the present findings demonstrate that the mitochondrion-dependent signalling pathway is activated in granulosa cells of atretic follicles, future studies may identify the Bid-like intracellular cross-talk molecule(s) mediating between the death receptor-dependent signalling pathway and the mitochondrion-dependent signalling pathway in granulosa cells of pig ovaries.

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References


Cecconi F (1999) Apaf1 and the apoptotic machinery Cell Death and Differentiation 6 1087–1098


Guthrie HD, Cooper BS, Welch GR, Zakaria AD and Johnson LA (1995) Atresia in follicles grown after ovulation in the pig: measurement of increased apoptosis in granulosa cells and reduced follicular fluid estradiol-17β Biological of Reproduction 52 920–927


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