Bid and Bax Are Involved in Granulosa Cell Apoptosis During Follicular Atresia in Porcine Ovaries

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Abstract. More than 99% of follicles undergo “atresia” during follicular development and growth. Follicular atresia is predominantly regulated by granulosa cell apoptosis. However, the intracellular signaling pathway of apoptosis in granulosa cells has not been revealed. In the present study, we examined changes in the expression of BH3-interacting domain death agonist (Bid) and Bcl-2-associated X protein (Bax), which are considered to promote the cell death ligand/receptor-mediated process in mitochondrion-dependent type II apoptosis, in porcine granulosa cells during atresia. Levels of mRNA and protein of Bid and Bax were determined by the reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting techniques, respectively. Levels of Bid and Bax mRNA and protein were markedly increased in granulosa cells of early atretic follicles compared with those of healthy follicles. In situ hybridization and immunohistochemical staining revealed that mRNA and protein of Bid and Bax were present in the granulosa cells, though only traces were found in healthy follicles; however, strong staining was noted in atretic follicles. These results indicate that Bid and Bax appear to be signal transduction factors in granulosa cells during follicular atresia and appear to play proapoptotic roles and confirm that the porcine granulosa cell is a mitochondrion-dependent type II apoptotic cell.

Key words: Bcl-2-associated X protein (Bax), BH3-interacting domain death agonist (Bid), Follicular atresia, Mitochondrion-dependent type II apoptotic cell, Pig ovary

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In mammalian ovaries, more than 99% of follicles degenerate at various stages of follicular growth and development [1]. The degeneration is explained, at least in part, by the apoptosis of granulosa cells [2–6]. In the early stages of follicular atresia in pig ovaries, biochemical and morphological characteristics typical of apoptosis, namely nuclear condensation, chromatin condensation and cell shrinkage, are observed 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 thecal layers [7, 8]. However, the intracellular signal transduction pathway of apoptosis in granulosa cells of pig ovaries is not well understood.

Selective apoptotic cell death is induced by cell death ligand/receptor systems, including Fas ligand (FasL; also called Apo-1 ligand or CD95 ligand) and Fas (also called Apo-1 or CD95), tumor necrosis factor (TNF)-α and TNF receptors (TNFRs) and TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors (DR4 and 5) [9–11]. The cell death ligand/receptor-mediated apoptotic signaling pathway is suggested to be as follows [12–18]: When trimerized cell death ligands bind with trimerized death receptors located on the cell membrane, the receptors are activated. An adaptor protein (Fas-associated death domain protein: FADD) binds with an activated receptor, and then procaspase-8 (an initiator caspase) is recruited and binds with FADD. Procaspase-8 is autotruncated and activated. To date, two types of death ligand/receptor-dependent signaling pathways have been found [19]. In the type I pathway, caspase-8 directly shortens procaspase-3 (an effector caspase). The activated caspase-3 then shortens caspase-3-activated DNase (CAD) [15]. The shortened CAD moves into the nucleus and cuts the genomic DNA leading to apoptosis [14]. In the type II pathway, BH3-interacting domain death agonist (Bid), which is known as a proapoptotic Bcl-2 family member and is located in the cytosol, is activated by caspase-8 cleavage upon engagement of cell surface death receptors [20–26]. The resulting 15-kDa C-terminal fragment (truncated Bid: tBid), which is also located in the cytosol, translocates to mitochondria and stimulates Bcl-2-associated X protein (Bax) [27–29]. Bax stimulated by tBid translocates to the mitochondrion where it undergoes oligomerization to induce the release of cytochrome c [30–33]. The proapoptotic activity of tBid is regulated by its interactions with pro-death Bax. The released cytochrome c binds with apoptotic protease-activating factor 1 (Apaf1) [34, 35]. The cytochrome c-Apaf1 complex binds with procaspase-9 (the complex is named as apotosome). Procaspase-9 is autoactivated and activated. The activated caspase-9 shortens a downstream caspase (procaspase-3). The activated caspase-3 shortens CAD, leading to apoptosis as in the case of the type I pathway. In the type II pathway, the mitochondrion provides a key amplification step. However, the exact roles of the mitochondrion in the apoptosis signaling pathway in follicular granulose cells have not been well understood.
Previously, we reported that granulosa cell apoptosis is regulated by inhibiting the activation of procaspase-8 and procaspase-9 in pig ovaries [36–40]. Cellular FLICE-like inhibitory protein (cFLIP), which is a homologue of procaspase-8 (also called FLICE) and acts as a competitive inhibitor [36], and X-linked inhibitor of apoptosis protein (XIAP), which acts as an inhibitor against procaspase-9 activation [40], are intracellular antiapoptotic factors. We also found decreased levels of procaspase-9 protein, increased levels of caspase-9 protein and increased proteolytic activity of caspase-9 during follicular atresia [41]. Based on these findings, we presumed that porcine granulosa cells undergo type II apoptosis. However, details of the intracellular apoptotic signaling pathway in porcine granulosa cells have not been confirmed. In the present study, to reveal the involvement of the mitochondrion-dependent apoptotic signaling pathway in granulosa cells, we examined changes in mRNA and protein levels of Bid and Bax, which transduce and amplify the apoptotic signal from activated caspase-8 to mitochondria and are essential factors for the type II pathway, in granulosa cells and changes in their distribution in follicles during atresia.

Materials and Methods

Preparation of follicular granulosa cells

As described previously [42–45], the ovaries were obtained from mature sows (more than 120 kg in body weight) at a local abattoir (Kasama, Japan). Each tertiary follicle with antrum, approximately 3 mm in diameter, was individually dissected from the ovaries under a surgical dissecting microscope (SZ40; Olympus, Tokyo, Japan). Each follicle was classified morphologically as healthy, early or progressed atretic and then opened using fine watchmaker-forceps to remove the granulosa layer and cumulus-oocyte complex. Follicular fluid was collected from the follicle using a 1-ml syringe (Terumo, Tokyo, Japan), separated by centrifugation at 3,000 g for 10 min at 4 °C, frozen and kept at –80 °C. After biochemical and histological analyses had been performed, 17β-estradiol (E2) and progesterone (P₄) levels were measured respectively using [125I]-RIA kits (Bio-Mérieux, Marcy-l’Etoile, France) to confirm the classification of the follicles. Follicles with a P₄/E₂ ratio of less than 15 were classified as healthy [46]. The granulosa cells were isolated by gentle pipetting with a Pasteur pipette, collected, washed three times in phosphate-buffered saline (PBS, pH 7.2) by centrifugation at 600 g for 5 min at room temperature (22–25 °C) and used for reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting analyses.

RT-PCR for caspase-8, caspase-9, Bid and Bax mRNAs

As previously reported [47–49], total RNA was extracted from each granulosa cell sample using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). Samples of cDNA were synthesized from equal amounts of the total RNA samples using a T-Primed First-Strand Kit (GE Healthcare, Little Chalfont, UK), according to the manufacturer’s protocols. The primers used for PCR were as follows: 5'-TCA AGT TCC TGA GCC TGG AC-3', forward, and 5'-GCA TGA CCC TGT AGG CAG A-3', reverse, for caspase-8 (GenBank accession number: AY519263); 5'-GAG CTT GAAG CAG CAC CAG-3', forward, and 5'-CTG GCT CTC TTG GAG GCT GCT TAC TGC CAG-3', reverse, for caspase-9 (AY522234); 5'-AGT GAT TCT AAG GTC AAC AAG G-3', forward, and 5'-CTG CAG GGA GAA CAG TGA A-3', reverse, for Bid (AY521564); 5'-ATG ATC GCA GCC GTG GAC ACG-3', forward, and 5'-ACG AAG ATG ATC GCA GCC GTG GAC ACG-3', reverse, for Bid (AJ606301); 5'-CTG GCT CTC TTG GAG GCT CAG-3', reverse, for porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AF017079, used as an intrinsic control). The expected PCR-product sizes of caspase-8, caspase-9, Bid, Bax and GAPDH were 254, 200, 598, 297 and 215 bp, respectively. PCR amplification was performed as follows: Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) and each primer pair were added to the cDNA mixture, mixed and denatured. The mixture was subjected to PCR in a thermal cycler (GeneAmp PCR System 9700; Invitrogen). The hot-start PCR profile was 94 C for 5 min, and then 30 cycles of 94 C for 30 sec, 53.5 C for 30 sec and 72 C for 1 min, followed by a final extension at 72 C for 2 min. To confirm the specific amplification of fragments of the expected size, the PCR products and molecular weight marker, a Ready-load 100-bp DNA Ladder (Invitrogen), were electrophoresed in 2% (w/v) agarose gels (Sigma-Aldrich, St. Louis, MO, USA), stained with ethidium bromide (Wako Pure Chemical Industries, Osaka, Japan). The stained gels were scanned with a digital fluorescence recorder (LAS-1000; Fujifilm, Tokyo, Japan), and the intensity of each mRNA band was quantified using the ImageGauge software (Fujifilm) on a Macintosh computer. Expression levels of each mRNA were normalized by the level of GAPDH mRNA. A representative PCR product was sequenced using an automatic DNA sequencer (ABI PRISM 310; Invitrogen) according to the manufacturer’s instructions.

Western blotting analysis of Bid and Bax proteins

As previously reported [42–45], for Western blotting, the protein fraction (10 µg/lane) prepared from each sample was separated by 10–20% (w/v) gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Atto, Tokyo, Japan) and transferred onto nitrocellulose membranes (Hybond-C; GE Healthcare). The membranes were stained with a 0.2% (w/v) Ponceau S solution (Serva Electrophoresis, Heidelberg, Germany) and immersed in a blocking solution [0.1 M Tris HCl (pH 7.6), 5% (w/v) skim milk, 0.05 M NaCl and 0.1% (v/v) Tween 20; Sigma] for 1 h. Each membrane was incubated with the appropriate primary antibody for 1 h at room temperature. For Bid and t-Bid, the membrane was incubated with a rabbit polyclonal anti-Bid antibody (550365; 1:2,500 dilution with blocking solution; BD Biosciences, Texarkana, TX, USA), for Bax, the membrane was incubated with a rabbit polyclonal anti-Bax antibody (N-20; 1:500 dilution with the blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and for GAPDH, the membrane was incubated with a rabbit polyclonal anti-GAPDH antibody (V-18; 1:200 dilution with the blocking solution; Santa Cruz Biotechnology). The membranes were washed with the blocking solution and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 25 C. For Bid and t-Bid, an HRP-conjugated polyclonal goat anti-rabbit IgG antibody diluted 1:5,000 (Dako Cytomation, Glos-
As in our previous reports [45], to make probes for *in situ* hybridization for *Bid* and *Bax* mRNA, conventional PCR amplification was performed. The primers used for *Bid* were 5'-GCG GAG ACA GAT TCT GAG AGC-3', forward, and 5'-AGT GTT CAG CCT CAG GA-3', reverse, and the expected size of the PCR product was 274 bp. The primers for *Bax* were 5'-GAC CGG GTG CCT CAG GA-3', forward, and 5'-CCC CAG TTG AAG TCT GAG AGC-3', reverse, and the product was 182 bp long. Platinum *Taq* DNA Polymerase (10,000 Unit/ml; Invitrogen) was added to the cDNA mixture and denatured. The mixture was subjected to PCR in a thermal cycler. The hot-start PCR profile for *Bid* and *Bax* was as follows: 96°C for 1 min, and then 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products for *Bid* and *Bax* were purified, and then used to make probes for *in situ* hybridization.

To visualize *Bid* and *Bax* mRNA by *in situ* hybridization, digoxigenin (DIG)-labeled antisense and sense cRNA probes were synthesized using a Lig' n Scribe Kit (Ambion, Austin, TX, USA) and a DIG RNA Labeling Kit (Roche Diagnostics, Rotkreuz, Switzerland) according to the instructions provided. Briefly, the ovaries obtained from mature sows at a local slaughterhouse were fixed with 20% (v/v) formalin and embedded in Histosec paraffin (Merck, Darmstadt, Germany). Serial sections 4-μm thick were cut on a microtome (SM2000R; Leica, Heidelberg, Germany), stretched in dimethyl pyrocarbonate (DMPC; Sigma) treated water, placed on silane-coated glass slides (Matsunami, Osaka, Japan), deparaffinized, and treated with 0.2 N HCl for 10 min at room temperature. After being washed with PBS, the sections were digested with proteinase-K (1 μg/ml; Sigma) in PBS at 37°C for 25 min, washed with PBS, immersed with 2 mg/ml of glycine in PBS for 20 min and washed well with PBS. They were then prehybridized with 10 mM Tris-HCl (pH 7.4) containing 600 mM NaCl, 1 × Denhardt’s solution and 50% (v/v) deionized formamide (Sigma) for 1 h at 25°C and hybridized with an antisense-DIG-*Bid* (or *Bax*) cRNA probe for 18 h at 45°C. Each probe (1 μg/ml) was diluted in 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetate, 600 mM NaCl, 1 × Denhardt’s solution, 10 mg/ml yeast tRNA, 10 mg/ml salmon testicular DNA, 5% (v/v) dextran sulfate and 50% (v/v) deionized formamide. The sections were washed with 2 × SSC [150 mM NaCl and 15 mM sodium citrate (pH 7.4)] for 1 h, 0.5 × SSC for 1 h and 0.2 × SSC for 30 min at 45°C and then equilibrated with 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (THS). They were treated with the blocking solution (Roche) for 1 h at room temperature and incubated with alkaline phosphatase (AP-) conjugated sheep anti-DIG antibody (1:500 diluted with the blocking solution; Roche) for 18 h at 4°C. After being washed with THS, the sections were rinsed with 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 100 mM MgCl₂ (THSM). They were incubated with THSM containing 0.4 mM nitro blue tetrazolium chloride (Sigma), 0.4 mM 5-bromo-4-chloro-3-indolyl-phosphate-4-toluidine salt (Sigma) and 1 mM levamisole (Sigma) and then incubated for 18 h at 4°C. The sections were washed with PBS, mounted with Histofine (Nichirei, Tokyo, Japan) and examined with a light microscope (BX51; Olympus). DMPC-treated water (Sigma) was used throughout the staining processes. As negative controls, serial sections were hybridized with a DIG-labeled sense cRNA probe or without any probe and incubated without the anti-DIG antibody. All the controls yielded negative results.

Histochemical detection of apoptotic cells, proliferating cell nuclear antigen (PCNA). *Bid* and *Bax*

As previously reported [45], apoptotic cells in porcine ovarian sections were detected histochemically by terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL). Briefly, formalin-fixed paraffin-embedded ovarian sections 4-μm thick were mounted on silane-coated glass slides, deparaffinized, rehydrated and washed in distilled water. Apoptotic cells were identified using an ApopTag Kit (Intergen, New York, NY, USA) according to the manufacturer’s instructions. The sections were counterstained with methyl green (Dako), dehydrated, mounted with Entellan (Merck) and examined by light microscopy. Positive and negative controls were included in each experimental run. As negative controls, sections were incubated without the terminal deoxynucleotidyl transferase and/or anti-DIG antibody.

To visualize the proliferating cells, PCNA was stained immunohistochemically as previously reported [45]. Briefly, the deparaffinized ovarian sections were immersed in methanol with 3% (v/v) H₂O₂ for 5 min. After being washed with 0.1 M Tris-HCl (pH 7.6), they were incubated with the primary antibody (anti-PCNA antibody: Epos anti-proliferating cell nuclear antigen; Dako) for 1 h at room temperature, washed with PBS and incubated with an HRP-conjugated anti mouse IgG antibody (Dako). The sections were stained with 0.1% (w/v) diaminobenzidine (Wako) and counterstained with methyl green. They were dehydrated, mounted with Entellan, and examined with a light microscope.

To visualize the location of *Bid* and *Bax* proteins, deparaffinized sections were immersed in methanol with 0.3% (v/v) H₂O₂ for 30 min to inhibit endogenous peroxidase activity. After a wash with PBS, appropriate nonimmunized normal serum solutions (Dako) were placed on the sections to block nonspecific binding. The sections were incubated with the appropriate primary antibody diluted in PBS containing 1% (w/v) bovine serum albumin (PBS-BSA; Sigma) for 1 h at room temperature. For *Bid* and tBid, a goat polyclonal anti-Bid antibody (AF860; diluted 1:500 with the blocking solution; R & D Systems, Minneapolis, MN, USA) was used. For *Bax*, a rabbit polyclonal anti-Bax antibody (N-20; diluted 1:500 with the blocking solution) was used. The sections were washed with PBS and incubated with the appropriate biotinylated secondary antibody (diluted 1:2,000 with PBS-BSA). For *Bid* and tBid,
the sections were incubated with an anti-goat rabbit IgG antibody (Chemicon; Temecula, California, USA) and for Bax protein, the sections were incubated with anti-rabbit goat IgG antibody (Chemicon). Immunoreactivity was visualized using a Vectastain Avidin-Biotin-Peroxidase Kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer’s protocols. The sections were counterstained with methyl green, dehydrated, mounted with Entellan and then examined with a light microscope.

Statistical analysis
An analysis of variance (ANOVA) with Fisher’s least significant differences test was carried out using the StatView-4.5 program (Abacus Concepts, Berkeley, CA, USA) on a Macintosh computer. Differences at P<0.05 were considered significant.

Results

Changes in caspase-8, Bid, Bax and caspase-9 mRNA levels in granulosa cells during follicular atresia

The levels of caspase-8, Bid, Bax and caspase-9 mRNA in porcine granulosa cells were estimated by RT-PCR. Representative photographs of PCR products are shown in Fig. 1A, and levels normalized to GAPDH mRNA are shown in Fig. 1B. Bid mRNA and Bax mRNA were expressed in healthy, early atretic and progressed atretic follicles. The highest levels of caspase-8, Bid, Bax and caspase-9 mRNA were found in granulosa cells of early atretic follicles. Lower levels of caspase-8, Bid and Bax mRNA were detected in those of healthy and progressed atretic follicles.

Distribution of Bid, tBid and Bax mRNA and protein and apoptotic and proliferating cells in ovarian follicles

No TUNEL-positive (apoptotic) cells and many PCNA-positive (proliferating) cells were observed in granulosa cell layers of...
Roles of Bid and Bax in Pig Granulosa Cell Apoptosis

Healthy follicles (Fig. 3A and D, respectively). Many TUNEL-positive cells and small numbers of PCNA-positive cells were observed in granulosa cell layers of early atretic follicles (Fig. 3B and E, respectively). In progressed atretic follicles, no layers of granulosa cells remained (Fig. 3C and F).

Weak labeling of Bid and Bax mRNA was detected in granulosa cell layers of healthy follicles (Fig. 3G and J, respectively). Stronger staining of Bid and Bax mRNA was observed in granulosa cell layers of early atretic follicles (Fig. 3H and K, respectively).

In granulosa cell layers of healthy follicles, weak reactions for Bid and tBid were seen (Fig. 3M), and Bax was detected (Fig. 3P). Stronger staining of Bid, tBid and Bax proteins in granulosa cell layers of early and progressed atretic follicles was demonstrated (Fig. 3N, O, Q and R, respectively).

The findings indicate that Bid and Bax mRNA levels and Bid, tBid and Bax protein levels increase in granulosa cell layers during follicular atresia.

Discussion

Apoptosis of granulosa cells, predominantly mediated by cell death ligand/receptor systems, is the first event in follicular atresia [2–6]. The intracellular signaling pathway of death ligand/receptor-dependent apoptosis has two signaling types, I and II, differing downstream of the activated caspase-8 [9–19]. In the type I pathway, procaspase-3 is directly activated by caspase-8 [14, 15]. However, in the mitochondrion-dependent type II pathway, caspase-8 stimulates the mitochondrion, and procaspase-9 and -3 are activated indirectly [14, 15]. Bid and Bax, Bcl-2 family members, act as proapoptotic factors in the type II pathway [20–33]. These Bcl-2 family members are expressed in the cultured ovarian cells of rodents [50, 51] and ovarian tissues of rodents [52, 53] and ruminants [54, 55]. However, it is not clear whether Bid and Bax are expressed in pig ovaries and involved in granulosa cell apoptosis in porcine follicles. In the present study, we revealed that mRNA and protein levels of Bid and Bax were higher in granulosa cells of early atretic follicles than in healthy follicles. Caspase-8, caspase-9 and caspase-3 have been detected in granulosa cells of atretic follicles [41]. Moreover, our histochemical data showed that apoptotic cells were present in granulosa layers of atretic follicles, but not in those of healthy follicles.

Based on the present results and our previous findings [2–8, 36–48], we consider the apoptotic signaling pathway in granulosa cells during follicular atresia in pig ovaries to be as follows. (1) A cell death ligand binds with its receptor, and an intracellular adaptor protein binds with the activated receptor [9–14]. (2) The receptor-adaptor protein complex recruits procaspase-8 (initiator caspase), which is then autoactivated [15]. (3) The activated caspase-8 shortens Bid, and then tBid activates Bax, which binds with outer membrane of the mitochondrion and is capable of forming a selective ion channel (mitochondrial permeability transition pore: PT pore) in membranes. [20–29]. (4) The activated Bax forms a PT pore in the mitochondrial membrane, and cytochrome c is released [30–33]. (5) Cytochrome c, Apaf1 and procaspase-9 form a complex (apoptosome), and then procaspase-9 is activated [34, 35]. (6) The activated caspase-9 activates procaspase-3, and the activated caspase-3 attacks CAD [15]. (7) The activated CAD moves into the nucleus, where it shows nucleolytic activity against genomic DNA, and finally apoptosis occurs [14].

We conclude that the porcine granulosa cell is a mitochondrion-dependent type II apoptotic cell and that proapoptotic proteins, Bid and Bax, have crucial roles in intracellular signal transduction and are involved in granulosa cell apoptosis, a key event of follicular selection, in pig ovaries. Further study is needed to reveal the exact mechanisms behind the activation of Bid and Bax, the regulation of
their expression and the release of cytochrome c from mitochondria.

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