X-linked inhibitor of apoptosis protein and active caspase-3 expression patterns in antral follicles in the sheep ovary

Hollian R Phillipps^{1,3}, Ilona C Kokay^{1,2}, David R Grattan^{1,2} and Peter R Hurst^{1,3}

¹Department of Anatomy, School of Medical Sciences and ²Centre for Neuroendocrinology, University of Otago, Dunedin 9016, New Zealand and ³Centre for Reproduction and Genomics, Invermay Agricultural Centre, Mosgiel 9053, New Zealand

Correspondence should be addressed to P R Hurst at Department of Anatomy, University of Otago, PO Box 913, Dunedin 9054, New Zealand; Email: peter.hurst@anatomy.otago.ac.nz

Abstract

X-linked inhibitor of apoptosis protein (XIAP) interacts with caspases to inhibit their activity, thereby providing a potential mechanism for regulation of granulosa cell apoptosis occurring during follicular atresia. The aim of this study was to determine the presence and localization of XIAP mRNA and protein content in the sheep ovary and compare these expression patterns with active caspase-3 protein in the same antral follicles. Romney ewe estrous cycles ($n=25$) were synchronized with 2–3 Estrumate injections and ovarian tissue collected during the luteal and follicular phases of the cycle. The presence of XIAP mRNA was confirmed by RT-PCR using laser capture microdissected ovarian cell samples. XIAP mRNA was subsequently localized by *in situ* hybridization histochemistry and XIAP and active caspase-3 protein visualized by immunohistochemistry. In antral follicles extensive XIAP localization was evident in both granulosa and thecal cells. In contrast, mRNA expression was widespread in granulosa cells and only detected in thecal tissue from a small proportion of antral follicles. Active caspase-3 and XIAP comparative expression analysis showed positive XIAP mRNA expression in all late luteal phase (day 14) follicles, despite varying levels of active caspase-3 protein. A proportion of follicular phase (days 15 and 16) follicles, however, showed an inverse expression relationship at the protein and mRNA levels in both granulosa and thecal tissue, as did XIAP protein in day 14 follicles. These results suggest high XIAP may prevent activation of caspase-3, thereby regulating follicular atresia in antral follicles and could potentially be utilized as a marker of follicular health.

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Introduction

Atresia, a degenerative process involving granulosa cell apoptosis, is the eventual fate of the majority of ovarian follicles during reproductive years (Hussein 2005). Apoptosis can be induced either within the cell mitochondria or by death inducing ligands, such as Fas ligand, binding to cell surface receptors. This leads to activation of a number of signaling pathways in which caspases are pivotal molecules (Hussein 2005). Caspases are cysteine proteases capable of cleaving target substrates at aspartic acid residues, promoting cellular breakdown. These proteases act either as initiators, such as caspase-9 or effectors (executioners), for example, caspase-3, depending on their position in the apoptotic signaling cascade. Caspases display a functional connection whereby upstream initiators can cleave and activate downstream effectors (Slee *et al.* 1999, Kumar 2007). Inhibition of caspase activation can occur at the level of zymogen conversion and downstream, averting active protease interaction with specific substrates, as reviewed by (Callus & Vaux 2007). Previous studies in

mice, rat, cow, and human ovaries have shown active caspase-3 is a common executor of apoptosis in antral staged follicles (Boone & Tsang 1998, Matikainen *et al.* 2001, Fenwick & Hurst 2002, Nicholas *et al.* 2005, Hurst *et al.* 2006). Consequently, this protein can be utilized as a reliable marker of the early stages of follicular atresia.

Inhibitor of apoptosis proteins (IAPs), initially identified during genetic screening of *p35* homologs during baculoviral infection, were subsequently found in additional species, including yeast, fruit fly, and humans (Crook *et al.* 1993, Hay *et al.* 1995, Roy *et al.* 1995, Duckett *et al.* 1996, Uren *et al.* 1999). These proteins are characterized by one or more baculoviral IAP repeat (BIR) zinc-binding domain(s) and found to be endogenous regulators of apoptosis (Clem & Miller 1994, Duckett *et al.* 1996, Hinds *et al.* 1999, Mace *et al.* 2009). There has been considerable debate concerning the underlying mechanism of this regulation. X-linked IAP (XIAP), cIAP1, and cIAP2 have been shown to bind caspases via their BIR2 or BIR3 domains (Deveraux *et al.* 1997, Roy *et al.* 1997); however, only XIAP has the ability to potentially inhibit enzymatic activity of caspases-3, -7, and -9

via these interactions (Sun *et al.* 2000, Shiozaki *et al.* 2003, Eckelman & Salvesen 2006). The mechanism and key residues responsible for tight XIAP/caspase binding have been characterized (Sun *et al.* 1999, Huang *et al.* 2001, Riedl *et al.* 2001, Scott *et al.* 2005). XIAP inhibits active caspase-3 and -7's active site by a two-site binding mechanism involving the BIR2 domain and preceding linker sequence (Chai *et al.* 2001, Riedl *et al.* 2001, Scott *et al.* 2005). Active caspase-9 inhibition is also achieved by two-site binding, however, this occurs independent of caspase-9's active site, via interaction of the BIR3 domain with monomeric procaspase-9's dimeric interface preventing homodimer formation (Shiozaki *et al.* 2003).

XIAP expression has been localized in the ovary of rats (Li *et al.* 1998, Lai *et al.* 2003, Lareu *et al.* 2003) and pigs (Cheng *et al.* 2008) and observed *in vitro* in ovarian cell types, including human ovarian surface epithelial (OSE) cells (Johnson *et al.* 2004) and bovine granulosa cells (Kayamori *et al.* 2009). A positive expression gradient during follicle development has been observed in pigs and rats, in which the highest levels occur at the antral follicle stage (Li *et al.* 1998, Cheng *et al.* 2008). Furthermore, comparative analyses in these species involving XIAP mRNA and/or protein expression levels and degree of follicular atresia as measured by the ratio of progesterone/estradiol-17 β in follicular fluid or by TUNEL expression have implicated XIAP as a potential key regulator of granulosa cell apoptosis (Li *et al.* 1998, Cheng *et al.* 2008). In rat granulosa cells, FSH has been shown to promote XIAP expression *in vivo* and *in vitro*, further supporting an anti-apoptotic role for XIAP in this cell type (Asselin *et al.* 2001, Wang *et al.* 2003). Moreover, XIAP can induce AKT upregulation in rat granulosa cells implicating involvement of the phosphatidylinositol 3-kinase signaling pathway, which is known to promote cell survival (Asselin *et al.* 2001).

The aim of this study was to determine the presence and localization of XIAP mRNA and protein and to investigate changes in XIAP levels in relation to follicular atresia using active caspase-3 protein expression as a marker of granulosa cell apoptosis in the sheep ovary. Sheep were utilized as they provide the opportunity for easy manipulation of reproductive cycles in a large animal model, contain ovaries with high follicle numbers at a range of different stages and like humans they predominantly undergo a single ovulation per cycle. Furthermore, mutations in important reproductive genes, such as *BMP15*, cause female infertility in both humans and sheep, but reduce ovulation rates in rodents (Shimasaki 2006). Consequently, sheep are an appropriate model to test the hypothesis that XIAP levels will be elevated in healthy antral follicles compared with atretic antral follicles and will show an inverse relationship with active caspase-3 protein expression in the same antral follicles.

Results

RT-PCR

RNA was extracted from whole ovary tissue and laser capture microdissection (LCM) isolated cell type samples collected on days 10, 14, and 16 of the estrous cycle. Presence of the β -actin (741 bp) transcript was used to confirm RT success. Then positive anti-Müllerian hormone (AMH; 342 bp) RT-PCR product bands were used as a granulosa cell-specific marker to confirm absence of contamination in isolated thecal, stromal, and luteal cells, respectively, before downstream reactions (Fig. 1A and B). XIAP expression was present, evident as a 357 bp product band in all isolated granulosa, thecal, stromal, and luteal cell LCM samples and whole ovary tissue extracts collected during the luteal phase (days 10 and 14) and follicular phase (day 16) of the estrous cycle (Fig. 1C–E).

In situ hybridization histochemistry for XIAP

A proportion of antral follicles showed positive XIAP mRNA expression in granulosa cells (Table 1 and Fig. 2A, B, and E–H). Hybridization to XIAP mRNA in thecal tissue was infrequent and granulosa

Figure 1 XIAP expression in ovarian tissue detected by RT-PCR. (A and B) Quality control analysis. A, AMH; B, β -actin. (A) Lane 1, granulosa cells; lane 2, thecal cells; lane 3, stromal cells; lane 4, luteal cells; lane 5, RNase-free water control. (B) Lane 1, granulosa cells; lane 2, granulosa cell RT negative; lane 3, thecal cells; lane 4, thecal cell RT negative; lane 5, stromal cells; lane 6, stromal cell RT negative; lane 7, luteal cells; lane 8, luteal cell RT negative; lane 9, RNase-free water control. RT-PCR analysis of XIAP transcripts in ovarian tissue collected days 10 (C), 14 (D), and 16 (E) of the estrous cycle. (C) Lane 1, granulosa cells; lane 2, thecal cells; lane 3, stromal cells; lane 4, luteal cells; lane 5, whole ovary tissue extract; lane 6, RNase-free water control; lane 7, blank. (D and E) Lane 1, granulosa cells; lane 2, thecal cells; lane 3, stromal cells; lane 4, luteal cells; lane 5, whole ovary tissue extract; lane 6, RNase-free water control. Oligonucleotide primer sets used are listed in Table 3.

Table 1 Comparison between active caspase-3 immunopositive granulosa cell numbers and the presence or absence of X-linked inhibitor of apoptosis protein (XIAP) mRNA expression in the same days 14, 15, and 16 antral follicles in the sheep ovary.

Follicle numbers	Follicle diameters (mm)	Disruption of granulosa cell layers	Number of active caspase-3 immunoreactive granulosa cells	XIAP expression	
				Granulosa cells	Thecal cells
(A) Day 14 antral follicles					
1	1.32	Yes	9	Yes	No
2	0.72	Yes	485	Yes	No
3	4.00	No	10	Yes	No
4	2.29	Yes	401	Yes	Yes
5	0.62	No	2	Yes	Yes
6	0.33	Yes	128	Yes	No
7	0.91	No	18	Yes	No
8	0.45	Yes	97	Yes	No
9	0.73	No	127	Yes	No
(B) Day 15 antral follicles					
1	0.58	Yes	502	No	No
2	0.83	Yes	13	No	No
3	3.50	No	8	Yes	No
4	1.19	No	0	Yes	Yes
5	0.35	Yes	0	No	No
6	3.75	Yes	47	No	No
(C) Day 16 antral follicles					
1	0.41	Yes	17	No	No
2	0.36	No	1	Yes	Yes
3	0.46	No	7	No	No
4	0.84	No	7	Yes	No
5	0.63	No	10	Yes	No
6	0.83	No	7	No	No
7	0.34	Yes	41	No	No
8	0.79	No	60	No	No

cells within the same antral follicles with antisense probe hybridization in thecal cells also showed positive expression (Table 1 and Fig. 2E–H). XIAP antisense probe hybridization, however, was not detected at earlier follicle stages, including primordial, primary, and preantral follicles. Variable levels of hybridization were apparent in positive antral follicles present in the same section plane at all three tissue collection time points during the estrous cycle (Fig. 2A and B). Furthermore, antral follicles containing granulosa and thecal cells with increased XIAP mRNA content showed changes in expression intensity across tissue layers (Fig. 2G and H). Granulosa cells closer to the basement membrane or in the theca interna showed higher XIAP levels compared with granulosa cells alongside the antral space or in the theca externa. The XIAP antisense probe hybridized to oocytes ($n=3$) within antral follicles containing high granulosa cell XIAP levels (Fig. 2E and F), however, no expression was evident in oocytes ($n=3$) within antral follicles showing minimal XIAP mRNA expression. No convincing positive mRNA expression was observed in the stroma or OSE, however, low hybridization was evident in luteal cells (Fig. 2C and D). Sections treated with the sense probe showed no specific hybridization in any cell type (Fig. 2I and J).

Immunohistochemistry for XIAP and active caspase-3

XIAP immunohistochemistry provided higher detection sensitivity than *in situ* hybridization histochemical studies as the protein was evident in follicles from the primary stage onwards and in thecal tissue surrounding a high proportion of antral follicles (Table 2 and Fig. 3A–E, 6A, B, E, and G). Expression was predominantly cytoplasmic in both granulosa and thecal cells and infrequent possible nuclear or perinuclear localization was apparent (Fig. 3F). Moreover, localization patterns did not change at any of the tissue collection time points during the estrous cycle. All antral follicles showed positive staining (Table 2) but levels of expression (as seen with mRNA levels) varied between follicles present in the same tissue section. Similar changes in expression intensity to that observed in *in situ* hybridization histochemical studies across granulosa and thecal cell layers from cells alongside the antral space to the theca externa was also apparent. Protein expression also localized to OSE cells (Fig. 3G), some stromal cells surrounding small follicles and luteal cells (Fig. 3H). No positive immunoreactivity was observed in negative or IgG control sections (Fig. 3I and J).

Active caspase-3 protein expression was detected primarily in granulosa cells in follicles from the early antral stage onwards (Fig. 4A–E). At the cellular level, its

Figure 2 Corresponding bright and dark field images showing *XIAP* mRNA localization. (A and B) High levels of positive hybridization for *XIAP* mRNA in granulosa cells in an antral follicle. Low thecal cell expression is also evident. A second antral follicle on the same section with lower *XIAP* expression in granulosa cells is shown in the superior aspect of the image. (C and D) Low hybridization to *XIAP* is evident in luteal cells (CL). (E and F) An increased signal is shown in an antral follicle oocyte, cumulus oophorus and surrounding granulosa and thecal cells. (G and H) Variable *XIAP* mRNA expression is shown in granulosa and thecal cells (indicated by a line with two arrowheads). Cells closer to the basement membrane and in central cell layers show elevated mRNA expression compared with cells adjacent to the antral space or in theca externa. Images (I and J) depict an antral follicle in a section treated with a sense probe. Scale bars A, B, I, and J, 200 μ m; C–H, 100 μ m. An, Oc, GC, TC, and St indicate antral space, oocyte, granulosa cells, thecal cells, and stroma respectively. Arrows in (G and H) indicate basement membrane.

localization was similar to *XIAP*, being predominantly cytoplasmic and a small number of cells showing nuclear/perinuclear expression (Fig. 4F and G). Two distinct active caspase-3 immunopositive granulosa cell localization patterns were apparent. In some follicles immunopositive cells were mostly located adjacent to the antral space (Fig. 4F), while in others expression was scattered more evenly throughout granulosa cell layers

(Fig. 4G). Occasional staining of single thecal cells was observed, however, only a small proportion of thecal cells (one to two immunopositive cells on a single section plane) showed active caspase-3 protein expression (Fig. 4E) and no signal was detected in oocytes or stroma. Furthermore, active caspase-3 immunopositive cells were detected in some corpora lutea and were predominantly located in the structure's periphery (Fig. 4H). No specific staining was observed on negative control slides and some IgG control sections showed a faint light pink background hue (Fig. 4I and J).

Comparison between *XIAP* mRNA and protein expression and active caspase-3 protein expression in days 14, 15, and 16 antral follicles

A semi-quantitative comparative analysis was undertaken on *XIAP* mRNA and active caspase-3 protein expression in $n=23$ antral follicles (0.3–4 mm in diameter). *XIAP* mRNA expression was detected in all late luteal phase (day 14) follicles studied, despite varied active caspase-3 immunoreactivity (Table 1 and Fig. 5A–F). In a proportion of follicular phase (days 15 and 16) follicles, however, *XIAP* mRNA showed an inverse relationship with active caspase-3 protein expression (Table 1 and Fig. 5G–L). Subsequent comparison of *XIAP* and active caspase-3 protein expression patterns in $n=22$ antral follicles (0.1–1.3 mm in diameter) demonstrated that the presence of active caspase-3 immunopositive granulosa cells did not always infer complete absence of *XIAP* expression in days 14 and 15 antral follicle granulosa cells (Table 2). A more consistent reduction in *XIAP* expression with increasing active caspase-3 immunopositive granulosa cells was observed in follicles collected on day 16 of the cycle (Table 2 and Fig. 6). *XIAP* protein expression in thecal tissue from these same follicles at all three collection points followed a consistent pattern of reduction becoming progressively localized to the interna layer with increasing active caspase-3 immunoreactivity and virtually absent when active caspase-3 protein levels were high (Fig. 6).

Discussion

This study aimed to test the hypothesis that *XIAP* levels will be elevated in healthy antral follicles compared with atretic antral follicles and will show an inverse relationship with active caspase-3 protein expression in the same antral follicles. Presence of *XIAP* mRNA was initially determined in whole ovary tissue extracts and subsequently by LCM of specific ovarian cell types. Positive *XIAP* mRNA and protein expression in ovarian follicles and the corpus luteum is consistent with findings from previous experiments in rats and pigs (Li *et al.* 1998, Wang *et al.* 2002, Lareu *et al.* 2003, Cheng *et al.* 2008). Evidence of positive *XIAP* expression at the protein level in stromal tissue surrounding small follicles, however, was not

Table 2 Comparison between active caspase-3 immunopositive granulosa cell numbers and the intensity of X-linked inhibitor of apoptosis protein (XIAP) immunoreactivity in granulosa and thecal cells from the same days 14, 15, and 16 antral follicles in the sheep ovary^a.

Follicle numbers	Follicle diameters (mm)	Disruption of granulosa cell layers	Number of active caspase-3 immunoreactive granulosa cells	XIAP expression	
				Granulosa cells	Thecal Cells
(A) Day 14 antral follicles					
1	0.24	No	2	+++	++
2	0.52	Yes	425	++	++
3	0.50	Yes	132	+	++
4	1.04	Yes	27	+	+
5	0.72	No	0	++	+++
6	0.13	No	1	++	++
(B) Day 15 antral follicles					
1	0.18	No	0	+++	+++
2	0.31	Yes	587	++	+
3	0.30	No	3	++	++
4	0.25	No	3	+++	+++
5	0.22	No	1	++	++
6	0.34	Yes	0	+	—
7	0.46	Yes	2	+	+
8	0.11	No	0	+++	++
9	0.24	No	2	+++	+++
10	1.32	No	6	+++	++
(C) Day 16 antral follicles					
1	0.44	No	2	+++	++
2	0.20	Yes	0	+	—
3	0.43	Yes	15	+	+
4	0.42	No	0	+++	+++
5	0.62	Yes	503	+	+
6	0.60	No	3	+++	+++

^a(—), Indicates no XIAP immunoreactivity; (+), indicates occasional XIAP immunoreactivity; (++) , frequent XIAP immunoreactivity; (+++), extensive XIAP immunoreactivity.

recorded in an earlier study investigating XIAP expression in the rat indicating a possible difference existing between rodent and ruminant species (Li *et al.* 1998).

The apparent absence of XIAP expression in primordial follicles suggests it acts downstream of initial recruitment during folliculogenesis, having a role specific to growing follicles. Moreover, XIAP immunopositive stromal cells observed surrounding primary follicles in this study suggests a potential role in recruitment and/or differentiation of thecal cells, which may be additional to its anti-apoptotic function. Involvement in thecal cell differentiation has also been suggested following observations of higher XIAP expression in rat thecal cells compared with granulosa cells in growing follicles (Lai *et al.* 2003). Furthermore, XIAP has been associated with differentiation of other cell types, including human monocytes, rat luteal cells and in regulating the rate of alveolar differentiation in the mouse mammary gland during late pregnancy (Lareu *et al.* 2003, Tamm *et al.* 2004, Olayioye *et al.* 2005). In this study, XIAP mRNA and protein localization in antral follicle granulosa cells collected during the follicular phase (days 15 and 16) of the cycle generally showed similar expression patterns. Moreover, positive expression observed in mural and cumulus granulosa cells and also thecal tissue in antral follicles supports findings in rat studies (Li *et al.* 1998, Lai *et al.* 2003).

An increase in XIAP expression associated with follicle maturation as shown in the rat ovary (Li *et al.* 1998), however, was not observed in this study. Immunoreactivity levels in follicles smaller than the preantral stage were not described in the rat study whilst here in sheep intense staining was detected in some small follicles. Variation in XIAP mRNA and protein expression intensity across cell layers, from granulosa cells alongside the antral space to the theca externa in antral follicles observed in this study suggests additional factors within the follicular fluid and/or stromal tissue immediately surrounding follicles may be contributing to XIAP's effect in the follicle.

Detection of XIAP expression in luteal cells in this study suggests a potential role in the development and maintenance of this structure, as has been proposed in rats (Lareu *et al.* 2003). Moreover, a gradual reduction in XIAP expression levels has been implicated as a potential regulatory mechanism for corpora lutea regression via apoptosis in rats following pregnancy (Lareu *et al.* 2003).

Variable XIAP expression levels in a proportion of antral follicles showing differing degrees of follicular atresia observed in this study has also been recorded in earlier studies in rats and pigs (Li *et al.* 1998, Cheng *et al.* 2008). Here in sheep active caspase-3 protein was utilized as an early atresia marker as *in vivo* and *in vitro* analyses have shown its expression to precede DNA

Figure 3 Immunohistochemical localization of XIAP. Positive immunoreactivity is denoted by red particulate stain (AEC). (A) Image of a whole ovary section (adjacent to that shown in Fig. 4A) showing the pattern of XIAP expression. Intense immunoreactivity is evident in the corpus luteum (CL). The black boxes indicate the location of follicles and luteal cells depicted in (B, C, E, and H) respectively. (B) An antral follicle with low XIAP expression in granulosa and thecal cell layers (compare with active caspase-3 protein expression in the same follicle Fig. 4B). Images (C–E) show positive XIAP immunoreactivity in granulosa cells in preantral follicles (C and D) and an early antral follicle (E; compare (C–E) with active caspase-3 protein expression in the same follicles in Fig. 4C–E). Immunoreactive thecal cells are also evident in (D and E). (F) A high magnification image showing predominant cytoplasmic localization of XIAP in both granulosa and thecal cell layers in an antral follicle. Arrows indicate examples of intense immunostaining. The OSE (G) and luteal cells (H) also showed positive XIAP immunoreactivity. Arrows in (G) indicate immunopositive cells. Images (I and J) depict antral follicles in negative and IgG control sections respectively. Scale bars A, 3000 µm; C and F, 20 µm; B, D, E, and H–J, 100 µm; and G, 50 µm. An, Oc, GC, TC, CL, and St indicate antral space, oocyte granulosa cells, thecal cells, corpus luteum, and stroma respectively.

fragmentation and the majority of apoptotic morphological changes, including cell shrinkage (Janicke *et al.* 1998, Matikainen *et al.* 2001, Hurst *et al.* 2006). Active caspase-3 localization observed in this study is consistent with expression patterns observed in mice and humans (Matikainen *et al.* 2001, Fenwick & Hurst 2002, Hurst *et al.* 2006). The levels of active caspase-3 protein expression are regulated by XIAP through inhibition, however, a number of factors also influence XIAP expression levels. The ring zinc finger domain present in XIAP has been implicated as playing a self-regulatory role as it exhibits E₃ ligase activity and facilitates selective apoptosis signal induced proteasome dependent self-degradation (Yang *et al.* 2000, Liston *et al.* 2001). Furthermore, a number of antagonists, such as second mitochondrial activator of caspases/direct IAP-binding protein with low pI (Smac/DIABLO; Chai *et al.* 2000, Wu *et al.* 2000) and high-temperature requirement A2/Omi (HtrA2/Omi) also regulate XIAP/caspase interactions. Consequently, XIAP synthesis, stability and

inhibition will lead to changes in active caspase-3 protein levels, which will affect the eventual fate of the follicles.

In healthy rat follicles XIAP is predominantly located in the nuclei of granulosa cells, whereas in atretic follicles it undergoes a localization shift and is primarily distributed in the cytoplasm (Li *et al.* 1998). A change in intracellular localization associated with atresia, however, was not observed in this study and may reflect species differences. Widespread XIAP expression in thecal tissue in healthy follicles, which becomes increasingly localized to interna layers and eventually disappears as atresia progresses in this study, is interesting. In cows, mice, and humans active caspase-3 immunopositive thecal cells are rarely observed (Matikainen *et al.* 2001, D'Haeseleer *et al.* 2006, Hurst *et al.* 2006), which is in agreement with results here in sheep. Moreover, the reason for elevated XIAP thecal expression in healthy follicles observed in several studies has not been addressed (Li *et al.* 1998, Asselin *et al.* 2001,

Figure 4 Immunohistochemical localization of active caspase-3 protein in the sheep ovary. Positive immunoreactivity is denoted by red particulate stain (AEC). (A) Image of a whole ovary section (adjacent to that shown in Fig. 3A) showing the pattern of active caspase-3 protein expression. The black boxes indicate the location of follicles and luteal cells depicted in (B, C, E, and H) respectively. (B) An antral follicle with numerous active caspase-3-positive granulosa cells (compare with XIAP expression in the same follicle, Fig. 3B). Images (C and D) show the absence of active caspase-3 protein in preantral follicles (compare with XIAP expression in same follicles Fig. 3C and D). An early antral follicle in (E) contains a single immunopositive thecal cell (indicated by arrow, compare with XIAP expression in the same follicle, Fig. 3E). Images (F and G) show higher magnification views of two different active caspase-3 immunopositive granulosa cell patterns in antral follicles. (F) Shows immunoreactivity predominantly presents adjacent to the antral space and (G) depicts a more even distribution throughout granulosa cell layers. (H) Luteal cells showing positive staining (examples indicated by arrows). Images (I and J) atretic antral follicles from negative and IgG control sections, respectively, showing absence of staining. Scale bars A, 3000 µm; C, F, and G, 20 µm; and B, D, E, and H-J, 100 µm. An, Oc, GC, TC, CL, and St indicate antral space, oocyte granulosa cells, thecal cells, corpus luteum, and stroma respectively.

Lai *et al.* 2003). XIAP may be acting as a thecal cell survival factor in response to the cytotoxic challenge presented by development of associated vasculature. Alternatively, low active caspase-3 activity associated with this tissue type may just be evidence of an evolutionary redundant mechanism as a study in rats found the endonuclease DNase I responsible for DNA fragmentation is absent in thecal cells or could represent an alternate mechanism of action, such as XIAP acting primarily at the level of caspase-9, although this requires

further investigation in ovaries (Boone & Tsang 1997). Low caspase-9 mRNA expression has only been detected in theca interna layers from advanced atretic pig follicles (Matsui *et al.* 2003).

Expression of XIAP mRNA in all late luteal phase (day 14) antral follicles with varying levels of active caspase-3 immunoreactivity suggests a potential redundant or unspecified role for XIAP may exist during this phase. Protein level observations indicate transcription of XIAP is differentially regulated at this time point. It may be that

Figure 5 Comparison between *XIAP* mRNA and active caspase-3 protein expression patterns. Positive active caspase-3 immunoreactivity is denoted by red particulate stain (AEC). (A–C and G–I) show corresponding bright (A and G) and dark (B and H) field images of extensive *XIAP* mRNA localization and corresponding low active caspase-3 protein expression levels (C and I) in the same day 14 (follicle 5, Table 1A; A–C) or 15 (follicle 3, Table 1B; G–I) antral follicles. Arrow in (C) indicates immunopositive cell, no positive staining is apparent in the field of view shown in (I). (D–F and J–L) depict corresponding bright (D and J) and dark (E and K) field images with an active caspase-3 comparison (F and L) showing high active caspase-3 expression levels in the same day 14 (follicle 2 Table 1A; D and E) or 15 (follicle 1, Table 1B; J and K) antral follicles. The granulosa cell layers in the day 14 antral follicles show high *XIAP* mRNA expression (D and E), despite the presence of numerous active caspase-3 immunopositive granulosa cells (F). Hybridization to *XIAP* in granulosa and thecal cell layers is by contrast absent in the day 15 follicle (J and K) that contains high active caspase-3 protein expression (L). Scale bars A–C and G–L, 50 μ m; D–F, 100 μ m. An, TC, and GC indicate antral space, thecal cells, and granulosa cells respectively.

antral follicles during the luteal phase retain the ability to regulate follicular fate in response to a specific stimulus, despite the presence of active caspase-3 immunopositive cells. Once in the follicular phase, however, irreversible selection for dominance associated with ovulation may block this. Consequently, despite varied results, a proportion of follicles sampled at this time point in this study showed an inverse *XIAP*/active caspase-3 expression relationship suggesting maintenance of *XIAP* might be a characteristic of selected follicles. Antral follicles in this study not showing an inverse expression relationship may be undergoing terminal atresia, by which time active caspase-3 expression is no longer detectable. This is in agreement with findings in

advanced atretic hamster follicles, in which a reduction in active caspase-3 expression was evident compared with early atretic follicles (Moffatt-Blue *et al.* 2006). Alternatively, as active caspase-3 protein expression occurs during the early stages of atresia, there may be a delay in downregulation of *XIAP* until active caspase-3 expression reaches a threshold concentration in a follicle. Furthermore, the variability in results obtained from the *XIAP*/active caspase-3 expression pattern analysis could be due to inclusion of both follicles able to respond to gonadotrophic hormones but not require this support for growth (gonadotrophin-responsive) and follicles reliant on FSH and LH for growth and development (gonadotrophin-dependent) which may

Figure 6 Comparison between XIAP and active caspase-3 protein immunolocalization in healthy and atretic day 16 antral follicles. Images (A–D) show a view of the entire follicle used in this comparison. The black boxes labeled (E–H), indicate the region of follicle wall depicted in the corresponding images (E–H). (E) Extensive XIAP was detected in both granulosa and thecal cell layers in the healthy antral follicle. (F) A section from the same healthy antral follicle had no active caspase-3 protein immunoreactivity. (G) Low XIAP expression is shown in granulosa cells in an atretic antral follicle. (H) A large number of active caspase-3 immunopositive granulosa cells are evident in the same follicle. Scale bars A–D, 200 μ m; E–H, 100 μ m. An, Oc, TC, and GC indicate antral space, oocyte, thecal cells, and granulosa cells respectively.

undergo atresia in response to differential stimuli (Scaramuzzi *et al.* 1993). Alternatively, unique properties associated with individual follicles at similar developmental stages may have also contributed to the variability. Therefore, future studies focused on specific antral follicle developmental time points encompassing both early and late stage atretic markers are likely to provide a clearer understanding of XIAP's role in atresia and its interaction with caspase targets.

In summary, this study has described in detail XIAP and active caspase-3 ovarian localization patterns at the end of the luteal phase and during the follicular phase in the sheep estrous cycle. XIAP mRNA and protein were detected in thecal tissue, a cell type displaying weak or trace active caspase-3 expression indicating either XIAP shows increased efficiency in active caspase-3 inhibition or XIAP could have alternate predominant caspase inhibition targets, such as caspase-9 in this cell type. Alternatively, XIAP may have a different role in these cells, such as thecal cell recruitment/differentiation, which requires further investigation. A proportion of antral follicles showing low active caspase-3 and high XIAP levels and *vice versa* recorded in this study suggests XIAP may act as a regulator of follicular atresia in the sheep ovary, however, further studies are required to characterize this interaction.

Materials and Methods

Animals

Ethics approval for all procedures involving animals was granted by the Invermay Animal Ethics Committee (AEC 11 484 and AEC 11 109) in accordance with the 1999 Animal Protection (Codes of Ethics Conduct) Regulations of New Zealand. Two- to seven-year-old Romney ewes ($n=25$) with no history of reproductive genetics were obtained from AgResearch Limited, Invermay, Mosgiel, Dunedin, New Zealand. Ewe estrous cycles were synchronized by 2 or 3 0.6–0.7 ml i.m. injections of Estrumate (active ingredients cloprostenol 250 μ g/ml, Southern Veterinary Supplies, Christchurch, New Zealand) given at 9- to 10-day intervals to promote luteolysis. Synchronization success was determined by running a teaser ram wearing a harness containing marker chalk with the ewes, confirming estrus (day 0) by visual observation of the presence of a mark on the ewe's hindquarter. All ewes had access to pasture and water *ad libitum* and were transported to an abattoir up to 72 h before being killed. Ewes were killed either by a lethal 10–20 ml i.v. injection of sodium pentobarbitone (Pentobarb 500, 500 mg/ml, National Veterinary Supplies Ltd, East Tamaki, Auckland, New Zealand) or by captive bolt and ovaries collected during the luteal phase (days 10 and 14) and follicular phase (days 15 and/or 16) of the subsequent natural cycle following synchronization.

Ovary processing

Ovaries were divided into halves or quarters and either snap frozen in liquid nitrogen for RT-PCR or frozen slowly in optimal cutting temperature embedding compound (Tissue-Tek, Sakura Finetek, Inc., Torrance, CA, USA) over liquid nitrogen cooled isopentane for laser capture microdissection (LCM), immunohistochemistry, and *in situ* hybridization histochemistry. Frozen tissue was stored at -80°C for up to 6 months before use. Adjacent sections (12 and $6\text{ }\mu\text{m}$) were cut in a cryostat at -22°C from tissue collected on days 14 ($n=3$, end of luteal phase), 15 ($n=3$, follicular phase), and 16 ($n=3$, follicular phase) of the cycle for *in situ* hybridization histochemistry and immunohistochemistry respectively. Sections were thaw mounted onto Superfrost plus-coated slides (ThermoFisher Scientific, Australia Pty Ltd, Scoresby, VIC, Australia) and stored at -80°C until use. For LCM, sections ($8\text{ }\mu\text{m}$) were cut in a cryostat at -22°C from ovaries collected on days 10 ($n=3$), 14 ($n=3$), and 16 ($n=3$) of the cycle and thaw mounted onto u.v. treated u.v. absorbent polyethylene terephthalate membrane frame slides (Leica Microsystems, Wetzlar, Germany). Sections were stored at -80°C overnight.

RNA extraction from whole ovary tissue

Total RNA was extracted from 50 mg fresh frozen whole ovary pieces collected during the luteal phase (days 10 ($n=3$) and 14 ($n=3$)) and follicular phase (day 16 ($n=3$)) of the cycle by a method based on that originally described by Chomczynski (Chomczynski 1993, Chomczynski & Sacchi 2006). Tissue was homogenized in TRIzol reagent (GibcoBRL, Grand Island, NY, USA) and phases separated using chloroform. RNA was precipitated in isopropanol and washed in 75% (v/v) ethanol. RNA was resuspended in Tris-ethylenediaminetetraacetic acid buffer and stored at -80°C . Total RNA concentration and purity was determined by u.v. spectrometry and integrity confirmed by agarose gel electrophoresis.

Laser capture microdissection

Sections were thawed at room temperature, postfixed in ice-cold acetone and stained with HistoGene (Arcturus, Mountain View, CA, USA). A Leica laser capture microdissection (LMD) system was used to cut out separate areas of granulosa, thecal, stromal, and luteal cells (if present), letting each cell type drop via gravity into $20\text{ }\mu\text{l}$ lysis buffer (Qiagen, Doncaster, VIC, Australia) in underlying caps of PCR tubes ($200\text{ }\mu\text{l}$ capacity). Once all cells of interest had been collected tubes were capped, centrifuged briefly and incubated at

-20°C for 1 h. RNA extraction was undertaken in accordance with the manufacturer's instructions using an RNeasy Micro Kit (Qiagen).

RT-PCR

RNA extracted from whole ovary pieces and LCM samples was reverse transcribed using Superscript III first-strand synthesis system for RT-PCR with random hexamers (Invitrogen, Auckland, New Zealand) according to the manufacturer's instructions. Resultant cDNA samples were stored at -20°C until use.

Oligonucleotide primer sets were designed using the Primer3 input website (<http://frodo.wi.mit.edu/>) for ovine *XIAP*, *AMH*, and β -actin using mRNA sequences obtained from either the NCBI GenBank database or kindly provided by Michelle French (AgResearch Limited) and custom made by Invitrogen (Table 3). PCR reactions were made up to $25\text{ }\mu\text{l}$ final volume consisting of $1\times$ Amplitaq Gold PCR master mix (Applied Biosystems, Melbourne, VIC, Australia), $1\text{ }\mu\text{l}$ whole ovary tissue cDNA or $2\text{ }\mu\text{l}$ LCM cDNA sample, 500 nm of each forward and reverse primer, and $10.5\text{ }\mu\text{l}$ (whole ovary tissue RNA extract) or $9.5\text{ }\mu\text{l}$ (LCM RNA extract) RNase-free water. These reactions were run in a thermocycler (Corbett Research, Mortlake, NSW, Australia) and subjected to initial denaturation at 94°C for 2 min, then repeated cycles (Table 3 for cycle number) of denaturation (94°C , 30 s), annealing (54°C (*XIAP*), 57°C (*AMH* and β -actin), 30 s), and extension (72°C , 30 s (*XIAP* and *AMH*), 45 s (β -actin)), followed by a final extension step at 72°C for 10 min. PCR products were run on a 1% (w/v) agarose gel and amplicon identity confirmed by DNA sequencing.

In situ hybridization for XIAP mRNA: 35-S labeled RNA probe preparation

The same *XIAP* primer set used for RT-PCR (Table 3), with T7 or SP6 promoter sequences incorporated onto the 5' ends was used to generate templates by RT-PCR using whole ovary cDNA. Resultant cDNA was purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. RNA probes labeled with $[35\text{-S}]$ UTP were produced by transcribing cDNA using a riboprobe *in vitro* transcription kit (Promega, Madison, WI, USA) with either T7 polymerase (antisense RNA probe) or SP6 polymerase (sense (control) RNA probe). Mini Quick spin RNA columns (Roche Applied Science, Indianapolis, IN, USA) were used to remove unincorporated nucleotides.

Table 3 Ovine oligonucleotide primer sets and number of amplification cycles used for RT-PCR.

Primer names	Sequences	Amplicon size (bp)	GenBank accession number	Cycle number
<i>XIAP</i> forward	5'-TTGTTGTGGTGGGAAGTTGA-3'	357	–	32 (whole tissue)
<i>XIAP</i> reverse	5'-TGTTGTTCCCAAGGGTCTTC-3'			38 (LCM samples)
<i>AMH</i> forward	5'-CAACACCTTGCTGAGGTTC-3'	328	NM_173890	40 (LCM samples)
<i>AMH</i> reverse	5'-AGTAACAGGGCTGGGGTCTT-3'			
β -Actin forward	5'-AACCGTGAGAAGATGACCCAG-3'	741	U39357	37 (whole tissue)
β -actin reverse	5'-CTCCTGCTTGCTGATCCACATC-3'			38 (LCM samples)

Hybridization

Sections were thawed at 55 °C for 5 min, postfixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min and washed twice in 0.5× saline-sodium citrate (SSC, 75 mM sodium chloride, 7.5 mM trisodium citrate dihydrate). Immersion in 1× Triethanolamine with 0.25% (v/v) acetic anhydride was undertaken to acetylate amino groups in the tissue, followed by two washes in 2× SSC and optimization of probe penetration using a graded ethanol series and chloroform. Sections were air-dried, covered with 100 µl hybridization buffer (50% (v/v) formamide, 0.3 M sodium chloride, 1× Denhardt's solution, 20 mM Tris, 5 mM EDTA, 10% (w/v) dextran sulfate, and 100 mM dithiothreitol) and prehybridized for 2 h at 42 °C. RNA probes (1.0×10^6 cpm in 120 µl hybridization buffer (final concentration)) were combined with tRNA (50 µg/section, Roche Applied Science) and denatured at 95 °C for 3 min before loading 20 µl antisense or sense probe/tRNA/hybridization buffer solution onto appropriate sections containing the prehybridization mixture. Sections were incubated at 55 °C for 24 h.

Posthybridization

Sections were washed in 2× SSC with 10 mM β-mercaptoethanol (BME)-1 mM EDTA and any single or unbound RNA removed by treatment with RNase A (20 µg/l, Sigma-Aldrich, St Louis, MO, USA) before a prolonged wash step in 0.1×SSC with 10 mM BME-1 mM EDTA for 2 h at 55 °C. Sections were dehydrated through a graded ethanol series, air-dried overnight and exposed to scientific imaging film (Kodak Biomax MR) for 7 days to generate autoradiographs. Each slide was dipped into LM-1 Hypercoat emulsion (Amersham Biosciences, Arlington Heights, IL, USA) and left to develop in a desiccated light safe box for 5 weeks at 4 °C. Sections were developed by immersing slides in Kodak Professional D19 developer (Eastman Kodak Company, Rochester, NY, USA) for 5 min, rinsing in tap water and then submerging in Ilford Hypam rapid fixer (Harman Technology Ltd, Cheshire, UK) for 10 min. Slides were transferred to tap water and sections counterstained with 1:4 strength Gill's haematoxylin, followed by dehydration in ethanol and drying at 42 °C. Sections were cleared in xylene and coverslipped using Vectamount (Vector Laboratories, Inc., Burlingame, CA, USA). Analysis for positive hybridization was undertaken using an Olympus AX70 research grade light microscope fitted with a spot RT5 megapixel camera under light and dark field illumination.

Immunohistochemistry for XIAP and active caspase-3

Sections were thawed at room temperature and postfixed in 0.5% (w/v) paraformaldehyde in 0.01 M PBS for 2 min. Immersion in 0.3% (v/v) hydrogen peroxide in methanol was used to quench endogenous peroxidases and sections covered with a blocking agent, 5% (v/v) goat or donkey serum (Sigma-Aldrich) in 0.01 M PBS for XIAP and active caspase-3, respectively, for 10 min. A mouse monoclonal anti-XIAP primary antibody (BD Biosciences, Franklin Lakes, NJ, USA;

Ca #610762, dilution 1:250) or a rabbit polyclonal active caspase-3 primary antibody (Cell Signaling Technology, Inc., Beverly MA, USA; Ca #9661, dilution 1:200) both diluted in 0.01 M PBS were loaded onto sections and incubated overnight at 4 °C. Controls included negative control sections covered with 0.01 M PBS only and IgG sections loaded with the appropriate IgG serum isotype, mouse IgG (Sigma, XIAP) and Rabbit IgG (Dako, Glostrup, Denmark; active caspase-3) diluted in 0.01 M PBS at the same concentration as the primary antibody used. Sections were incubated with either a goat biotinylated anti-mouse IgG (XIAP) or donkey biotinylated anti-rabbit IgG (active caspase-3; Amersham Biosciences) diluted 1:200 in 0.01 M PBS for 30 min. This was followed by incubation with streptavidin-biotinylated HRP 1:100 in 0.01 M PBS for 30 min. Sections were developed using a 3-amino-9-ethylcarazole chromogen kit (Sigma-Aldrich) in accordance with the manufacturer's instructions and counterstained with 1:4 Gill's haematoxylin. Slides were coverslipped using 90% (v/v) glycerol in 0.01 M PBS. Positive immunoreactivity in sections was analyzed using an Olympus AX70 research grade light microscope fitted with a spot RT5 megapixel camera.

Semi-quantitative ovarian follicle analysis of in situ hybridization histochemistry for XIAP and immunohistochemistry for XIAP and active caspase-3

Ovarian follicle stages were classified based on the system described by (Lundy *et al.* 1999). In brief, follicles were classed as: primordial, consisting of a single layer of flattened granulosa cells; primary, containing one layer of cuboidal granulosa cells; preantral, two to five granulosa cell layers with no antral space and encircled by thecal tissue; antral follicle, containing an antral space enclosed in granulosa cell layers and surrounded by thecal tissue. Section planes containing each antral follicle with a visible oocyte and nucleolus were not always available. This meant the diameter of each follicle was determined by averaging two measurements taken from the basement membrane set at right angles to each other using a low powered image of the largest available cross section. Active caspase-3 immunopositive granulosa cell numbers were recorded for each antral follicle also being examined for the presence or absence of XIAP mRNA and intensity of protein expression. A mean count of immunopositive cell numbers present in three different sections containing the largest cross sections available for each follicle were analyzed. Resultant active caspase-3 immunopositive cell counts and morphological observation of the presence or absence of granulosa cell layer disruption were used to assess the degree of atresia in each follicle.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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