AKT is involved in granulosa cell autophagy regulation via mTOR signaling during rat follicular development and atresia

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Abstract

In this study, we examined whether granulosa cell autophagy during follicular development and atresia was regulated by the class I phosphoinositide-3 kinase/protein kinase B (AKT) pathway, which is known to control the activity of mammalian target of rapamycin (mTOR), a major negative regulator of autophagy. Ovaries and granulosa cells were obtained using an established gonadotropin-primed immature rat model that induces follicular development and atresia. Autophagy was evaluated by measuring the expression level of microtubule-associated protein light chain 3-II (LC3-II) using western blots and immunohistochemistry. The activity of AKT and mTOR was also examined by observing the phosphorylation of AKT and ribosomal protein S6 kinase (S6K) respectively. After gonadotropin injection, LC3-II expression was suppressed and phosphorylation of AKT and S6K increased in rat granulosa cells. By contrast, gonadotropin withdrawal by metabolic clearance promoted LC3-II expression and decreased phosphorylation of AKT and S6K. In addition, in-vitro FSH treatment of rat granulosa cells also indicated inhibition of LC3-II expression accompanied by a marked increase in phosphorylation of AKT and S6K. Inhibition of AKT phosphorylation using AKT inhibitor VIII suppressed FSH-mediated phosphorylation of S6K, followed by an increase in LC3-II expression. Furthermore, co-treatment with FSH and AKT inhibitor increased the levels of apoptosis and cell death of granulosa cells compared with the single treatment with FSH. Taken together, our findings indicated that AKT-mediated activation of mTOR suppresses granulosa cell autophagy during follicular development and is involved in the regulation of apoptotic cell death.

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Introduction

Follicular atresia is an important selective process during mammalian ovarian follicle development (Byskov 1979, Hirshfield 1991) and is believed to involve granulosa cell death by apoptosis, a form of programmed cell death (PCD; Boone et al. 1995, Tilly et al. 1995, Hsu et al. 1996, Kim et al. 1999). However, some studies have reported that nonapoptotic forms of PCD such as autophagy can be induced in human granulosa cells (Duerrschmidt et al. 2006, Serke et al. 2009, Vilser et al. 2010). We recently reported that autophagy is induced mainly in the granulosa cells and promotes apoptotic cell death (Choi et al. 2010, 2011a). These results suggest that autophagy is directly involved in follicular atresia and plays an important role in the regulation of apoptotic granulosa cell death during folliculogenesis. Analyses of the molecular events during granulosa cell autophagy are pivotal to our understanding of how the massive cell death of granulosa cells is controlled during follicular atresia. However, the molecular pathways that regulate the induction of autophagy in granulosa cells during ovarian follicular development and atresia are not fully understood.

In many normal and cancer cells, mammalian target of rapamycin (mTOR) has been found to be a major negative regulator of autophagy and its activity is mainly regulated by activating signals such as the class I phosphoinositide-3 kinase (PI3K)/protein kinase B (AKT) pathway (Corc e et al. 2009, Chen & Karantza 2011). Indeed, the activation of AKT via an enhanced PI3K/AKT pathway inactivates mTOR repressor tuberous sclerosis complex 2, thus leading to activation of mTOR and subsequent inhibition of autophagy (Li et al. 2009, Qin et al. 2010, Zha et al. 2011). Thus, the PI3K/AKT pathway plays an important role in the regulation of autophagy induction by controlling mTOR activity. Furthermore, recent studies have demonstrated that this pathway can be triggered by various stimuli including growth factors (Jia et al. 2006, Wu et al. 2009) and hormones (Yu et al. 2010, Arumugam et al. 2012), although it was originally recognized as a response to nutrient deprivation. In granulosa cells, gonadotropin treatment activates the PI3K/AKT pathway, which also increases mTOR activity.
(Alam et al. 2004, Hunzicker-Dunn et al. 2012). The PI3K/AKT pathway may therefore also be involved in the regulation of granulosa cell autophagy via control of mTOR activity during follicular development and atresia, as gonadotropin is known to suppress granulosa cell autophagy in follicular development (Choi et al. 2010). However, there is also a report suggesting that mTOR activation by gonadotropin may not be controlled by the PI3K/AKT pathway in rat granulosa cells (Kayampilly & Menon 2007), and there is no direct evidence of the involvement of the PI3K/AKT pathway through mTOR in granulosa cell autophagy regulation.

Here, we evaluated the involvement of the PI3K/AKT pathway through mTOR in the autophagy of granulosa cells to determine the molecular pathways regulating autophagy induction of granulosa cells during follicular development and atresia.

Materials and methods

Animal treatment

Animal experimentation protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University School of Medicine. Immature female Sprague–Dawley rats at 21–23 days of age were injected i.p. with 15 IU equine chorionic gonadotropin (eCG; Sigma Chemical Co.) to induce ovarian follicular development and atresia (Dhanasekaran & Moudgal 1989, Hughes & Gorospe 1991). This treatment induces atresia (histologically and biochemically) in large antral and preovulatory follicles resembling those in animals after gonadotropin deprivation, hypophysectomy, and pentobarbital administration. In addition, ovulation (on day 3) and the presence of corpus lutea (on days 4 and 5) were rarely evident (<5%) in these experimental animals. Rats were killed by cervical dislocation on days 0, 1, 2, 3, 4, and 5 after gonadotropin treatment, and the ovaries were excised. After removal of connective tissues, the ovaries were briefly washed in PBS to remove excess blood and were either immediately fixed in 10% neutral buffered formalin for histological processing or used for isolation of granulosa cells by follicle puncture.

Granulosa cell collection and culture

Ovaries were excised 0, 1, 2, 3, 4, and 5 days after eCG injection and placed in DMEM/F12 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL), 10 mg/ml streptomycin sulfate (Sigma Chemical Co.), and 75 mg/ml penicillin G (Sigma Chemical Co.). Granulosa cells were harvested by follicle puncture using a 25-gauge needle. After follicle puncture, granulosa cells were suspended in the appropriate solution for immunoblotting.

For in vitro culture of granulosa cells under serum-free conditions, ovaries were collected 48 h after eCG injection (15 IU, i.p.), and granulosa cells were collected by follicle puncture as described earlier. The cells were seeded in 10-cm culture dishes or six-well plates, and allowed to attach overnight. The next morning, in order to evaluate the effects of FSH treatment on AKT, mTOR activity, and autophagy induction, cells were incubated with serum-free media supplemented with FSH (50 ng/ml, Sigma Chemical Co.) for 0, 30, 60, and 120 min. In addition, granulosa cells were cultured in serum-free media supplemented with FSH (50 ng/ml) and AKT inhibitor (5 μM Akt inhibitor VIII, Sigma Chemical Co.) for 30 min to inhibit AKT activity. Treatments were stopped by removing the media, and the granulosa cells were scraped to extract the proteins or fixed for immunofluorescence and electron microscopy analysis.

Western blot analysis

During autophagy, light chain 3 (LC3), which is widely used as an autophagic marker, is converted from LC3-I to LC3-II; LC3-II then becomes localized to isolated membranes and autophagosomes (Kabeya et al. 2000, 2004) and the amount of LC3-II expressed is correlated with the number of autophagosomes (Nara et al. 2002). We therefore measure the expression level of LC3-II protein by western blot analysis to evaluate autophagy of granulosa cells. AKT activity is determined by measuring the phosphorylated active form of AKT (Kandel & Hay 1999). mTOR activity was also evaluated by analyzing the phosphorylation of its substrate, ribosomal protein S6 kinase (S6K; Sarbassov et al. 2005). The freshly isolated and cultured granulosa cells were lysed with ice-cold radioimmunoprecipitation assay buffer that was supplemented with a protease inhibitor cocktail (Sigma Chemical Co.). Cell lysates were incubated on ice for 30 min to completely solubilize cellular proteins, followed by centrifugation (13 000 g, 4 °C, 30 min). The whole-cell lysates (20 μg/lane) were separated by SDS–PAGE and transferred to a PVDF membrane (Bio-Rad). After blocking nonspecific binding sites with 5% skim milk, the membrane was treated with rabbit polyclonal antibodies to LC3 (diluted 1:1000; Novus Biologicals, Littleton, CO, USA), total and phosphorylated AKT (Ser473) (diluted 1:1000 and 1:1000; Cell Signaling Technology, Boston, MA, USA respectively), and total and phosphorylated S6K (Ser235/236) (each diluted 1:1000; Santa Cruz Biotechnology) overnight at 4 °C. The immunoreactive bands were visualized by incubation with HRP-conjugated goat anti-rabbit IgG (diluted 1:5000; Santa Cruz Biotechnology) at room temperature (RT) for 1.5 h. Peroxidase activity was visualized by ECL (Amersham Pharmacia Biotech). Integrated optical intensities of the immunoreactive protein bands were quantified by imaging (Gel Doc 2000, Bio-Rad) and the analysis software Quantity One, version 4.0.3 (Bio-Rad). Expression of LC3-II was normalized to β-actin, while phosphorylated AKT and S6K were normalized to total AKT and S6K respectively.

Immunohistochemistry

Paraffin-embedded whole ovarian sections were deparaffi-

cinated, rehydrated, and placed in a steamer for 30 min in 10 mM citric buffer for antigen retrieval. Endogenous peroxide was reduced by incubation of the sections in 3% H2O2 for 30 min. Nonspecific binding was blocked with 5% BSA (Sigma Chemical Co.) in PBS for 30 min. After washing, sections were
incubated overnight at 4 °C with anti-cleaved caspase-3 rabbit polyclonal antibody (diluted 1:100; Cell Signaling Technology), anti-LC3 rabbit polyclonal antibody (diluted 1:500; Cell Signaling Technology), phosphorylated AKT rabbit polyclonal antibody (diluted 1:50; Cell Signaling Technology), or phosphorylated S6K rabbit polyclonal antibody (diluted 1:50) followed by incubation with a biotinylated secondary antibody (DAKO, Glostrup, Denmark) for 1 h at a dilution of 1:2000. After incubation with a streptavidin–peroxidase conjugate, antibody complexes were visualized with diaminobenzidine tetrahydrochloride chromogen. The sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls lacking primary antibody were processed on adjacent tissue sections.

**Immunofluorescence staining**

Granulosa cells were cultured on sterilized glass coverslips, fixed with 4% paraformaldehyde, and blocked with 0.1% BSA in PBS. Cells were incubated with anti-LC3 rabbit polyclonal antibody (diluted 1:400), phosphorylated with AKT mouse polyclonal antibody (diluted 1:200; Novus Biologicals) in PBS and reacted with Alexa 568- and 488-conjugated secondary antibodies (diluted 1:5000; Vector Laboratories, Burlingame, CA, USA). Finally, slides were mounted in mounting media (Vector Laboratories), and images were captured with a confocal microscope (Bio-Rad).

**Transmission electron microscopy analysis**

To identify autophagic vacuoles at the ultrastructural level, granulosa cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 45 min at 4 °C, rinsed in cacodylate buffer, postfixed in 1% OsO4 in cacodylate buffer, dehydrated, and embedded in Epon. Ultrathin sections were briefly contrasted with uranyl acetate and photographed with a transmission electron microscope (TEM; Hitachi 7100; Hitachi). For quantification of autophagosomes, the number of autophagosomes in the target cell sections was counted from at least ten randomly chosen TEM fields.

**Assessment of granulosa cell apoptosis**

The percentage of apoptotic cells was determined by Annexin-V–FITC Apoptosis Detection Kit (BD Biosciences, Becton Drive Franklin Lakes, NJ, USA) The cells were collected and washed with 1 × PBS and stained by Annexin V and propidium iodide (PI) in binding buffer for 15 min at RT in the dark. The cells were observed accordingly with a FACSaria flow cytometer (BD Biosciences, Heidelberg, Germany). At least 10 000 cells were analyzed in each treatment.

**Assessment of granulosa cell death**

Granulosa cell death was determined using the Trypan blue exclusion test (Trypan blue stains dead cells blue, but does not permeate living cell membranes). A 0.4% solution of Trypan blue (Sigma Chemical Co.) was added to 0.1 ml cell suspension and mixed thoroughly. The mixture was allowed to stand for 10 min at RT and then centrifuged at 500 g for 10 min at 4 °C. Supernatants were discarded, and pellets were resuspended in 0.1 ml of serum-supplemented media. A small droplet from each tube was transferred to a hemacytometer and observed under a microscope. Cells that stained blue were considered dead.

**Statistical analysis**

Cell death, apoptosis, LC3-II expression, the phosphorylation of AKT and S6K proteins and number of autophagosomes were reported as the mean (±S.E.M.). Statistical analysis was performed using ANOVA. Significant differences between the treatment groups were determined by Duncan’s multiple range tests. Statistical significance was inferred at a P value <0.05.

**Results**

**Autophagy induction and the phosphorylation of AKT and mTOR in granulosa cells during follicular development and atresia**

We used an established invivo model for the induction of follicular development and atresia in immature rats to determine the involvement of the PI3K/AKT pathway and mTOR in regulation of granulosa cell autophagy during folliculogenesis. As shown in Fig. 1A and B, LC3-II (17 kDa) expression in granulosa cells decreased significantly at 1 and 2 days after eCG injection compared with granulosa cells of immature rats not exposed to exogenous gonadotropin (day 0, control; P<0.05). LC3-II expression, however, increased significantly on day 3 compared with days 1 and 2 and these elevated expression levels were maintained until day 5 (P<0.05). By contrast, the phosphorylation level of AKT and S6K increased significantly after eCG injection (day 1) and decreased on days 3, 4, and 5 (P<0.05; Fig. 1A and B).

We also examined the co-localization of cleaved caspase-3, LC3, phosphorylated AKT, and S6K in eCG-primed immature rat ovaries. Based on the morphological condition of the granulosa cell layers and the granulosa cell apoptosis, follicles were classified into healthy and atretic follicles. Healthy follicles were defined as those with intact and well-organized multilaminar granulosa cell layers and weak cleaved caspase-3 staining of granulosa cells (Fig. 2IA and IIA), whereas atretic follicles were determined by disorganized granulosa cells, shrunken granulosa cells, and intense cleaved caspase-3 staining (Fig. 2IE and IIE). Granulosa cell layers of healthy early/medium and large antral follicles stained weakly for the LC3 protein (Fig. 2IB and IIB) and showed intense phosphorylated AKT and S6K immunoreactivity (Fig. 2IC, ID, IIC and IID). By contrast, granulosa cells with intense LC3 immunoreactivity (Fig. 2IF and IIF) showed very weak and/or partially distributed staining for phosphorylated LC3-II.
AKT and S6K in atretic early/medium and large antral follicles (Fig. 2IG, IH, IIG and IIH).

**FSH inhibits granulosa cell autophagy through an increase in AKT followed by mTOR activation**

To determine the effect of FSH on autophagy induction, the PI3K/AKT signaling pathway, and mTOR activity, granulosa cells were treated with or without FSH (50 ng/ml), and expression of LC-II and phosphorylation of AKT and S6K was evaluated. FSH treatment significantly increased AKT and S6K phosphorylation at 30, 60, and 120 min, with the maximum response seen at 30 min (Fig. 3A and B). By contrast, LC3-II expression decreased significantly after FHS treatment (Fig. 3B; \( P \leq 0.05 \)).

Next, we examined the endogenous expression of phosphorylated AKT and the subcellular localization of endogenous LC3 using immunofluorescence staining. Phosphorylated AKT and endogenous LC3 were easily detected as green and red fluorescence, respectively, in the cultured granulosa cells (Fig. 3C). In the granulosa cells cultured in the absence of FSH, phosphorylated AKT stained weakly (Fig. 3C, p-AKT, top) and, punctuated LC3-II structures accumulated in the cytoplasm (Fig. 3C, LC3, top). Conversely, when cells showed intense immunoreactivity for phosphorylated AKT due to FSH treatment (Fig. 3C, p-AKT, bottom), few punctuate LC3-II structures were detected throughout the cytoplasm (Fig. 3C, LC3, bottom).

We also examined the effect of AKT inhibition on the level of phosphorylated S6K and LC3-II expression to confirm whether the increased AKT activity affected mTOR and autophagy induction in granulosa cells. FSH-stimulated AKT was completely abolished by the addition of AKT inhibitor, accompanied by a decrease in S6K phosphorylation and increased LC3-II expression (\( P \leq 0.05 \); Fig. 4A and B). To confirm this finding, we took TEM images of granulosa cells cultured in a FSH-added condition with or without AKT inhibitor (Fig. 4C). Autophagic structures are characterized by the presence of multiple autophagosomes, which are double-membranous vacuoles containing engulfed cytoplasmic materials. Some autophagosomes were evident in granulosa cells cultured without AKT inhibitor (Fig. 4C, left). When granulosa cells were cultured with AKT inhibitor, however, the number of autophagosomes increased compared with granulosa cultured without AKT inhibitor (Fig. 4C, right). This change represented approximately a threefold increase in the mean number of autophagosomes per cell section (Fig. 4D).

**Apoptotic cell death of granulosa cells is induced with inhibition of AKT activity**

To determine the effects of AKT-mediated autophagy on apoptotic cell death, granulosa cells were treated with or
without FSH and AKT inhibitors, and granulosa apoptosis and cell death were examined by Annexin V/PI and Trypan blue staining respectively. As shown in Fig. 5, the rates of apoptosis and cell death in the FSH-treated granulosa cells were lower than those of granulosa cells cultured in the absence of FSH. By contrast, FSH-inhibited apoptosis and cell death were significantly increased by AKT inhibition ($P<0.05$).

**Discussion**

In many cell systems, autophagy is known to be regulated by the PI3K/AKT pathway leading to activation of the major negative regulator of autophagy, mTOR (Fujiwara et al. 2007, Degtyarev et al. 2009). Granulosa cell autophagy has been recognized as the cellular mechanism responsible for follicular atresia by affecting apoptotic cell death (Choi et al. 2010, 2011a). However, the role of the PI3K/AKT pathway and mTOR in the regulation of granulosa cell autophagy has remained unclear. Here, we examined the involvement of the PI3K/AKT pathway and mTOR in the regulation of granulosa cell autophagy by using an established gonadotropin-primed immature rat model inducing ovarian follicular development and atresia. Gonadotropins are important survival factors leading to selection and survival of the growing follicles during follicular development (Asselin et al. 2000). We previously demonstrated *in vivo* downregulation of granulosa cell autophagy in the presence of gonadotropins during follicular...

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**Figure 2** Cleaved caspase-3, LC3, phosphorylated AKT (p-AKT), and p-S6K proteins immunostained on adjacent sections of healthy (A, B, C, and D) or atretic (E, F, G, and H) early/medium (I) and large (II) antral follicles from immature rats 0–5 days after eCG injections (15 IU, i.p.). GC, granulosa cell; TC, theca cell. Bars = 50 μm.
In this study, we confirmed the inhibitory effect of eCG on granulosa cell autophagy and showed that eCG induced AKT and S6K phosphorylation in granulosa cells in vivo. By contrast, eCG withdrawal caused by metabolic clearance (days 3, 4, and 5) promoted granulosa cell autophagy with decreased phosphorylation of AKT and S6K. Thus, activation of AKT and mTOR may be key events in the inhibition of granulosa cell autophagy during follicular development, whereas inhibition of AKT and mTOR...
promoted granulosa cell autophagy to induce follicular atresia. This hypothesis was confirmed by colocalization of cleaved caspase-3, LC3, and phosphorylated AKT and S6K proteins in eCG-primed immature rat ovaries, because granulosa cells were intensely stained for LC3 with decreased immunoreactivity for phosphorylated AKT and S6K staining in atretic follicles showed intense cleaved caspase-3 staining. These in vivo findings suggest that AKT activity is involved in the regulation of granulosa cell autophagy through the control of mTOR activity during follicular development and atresia.

Other evidence for the involvement of AKT and mTOR in regulating granulosa cell autophagy came from the finding that high levels of phosphorylated AKT and S6K were present in granulosa cells in vitro under FSH-treated conditions, which is known to prevent autophagy induction of granulosa cells (Choi et al. 2010). This finding is supported by our immunofluorescence images showing accumulation of LC3-II combined with a decrease in phosphorylated AKT (Fig. 3C). We also demonstrated that AKT inhibitor not only decreased the FSH-mediated upregulation of mTOR activity but also increased autophagy, suggesting that mTOR activation and autophagy induction are under the influence of AKT activity in rat granulosa cells. Furthermore, our TEM images provided direct evidence of the increase in autophagy induction in AKT inhibitor-treated granulosa cells; ultrastructural changes typical of autophagy were visible, such as the increased numbers of autophagosomes within cells. Taken together, our in vitro results also suggest direct involvement of AKT in regulation of mTOR activity, and this event may be the main mechanism for controlling autophagy induction in rat granulosa cells.

Autophagy is known to induce apoptotic cell death by the accumulation of autophagosomes in ovarian cells, including granulosa cells (Choi et al. 2010) and luteal cells (Choi et al. 2011b). In addition, inhibition of the PI3/AKT pathway stimulates granulosa cell apoptosis (Asselin et al. 2001). Therefore, the high levels of AKT phosphorylation in the FSH-treated granulosa cells observed in our study may prevent these cells from undergoing apoptotic cell death by inhibiting autophagy. This hypothesis is supported by the observation that the FSH-induced decrease in apoptosis and cell death was reversed by co-treatment with FSH and AKT inhibitor. Thus AKT activity plays a pivotal role in the regulation of apoptotic granulosa cell death by controlling autophagy induction.

In conclusion, granulosa cell autophagy is inhibited by increased AKT activity followed by mTOR activation during follicular development, which suppresses apoptotic cell death.

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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Figure 5 The effects of AKT inhibition on apoptosis and cell death of granulosa cells. Granulosa cell apoptosis and cell death were examined by Annexin V/IP and Trypan blue staining respectively. (A) Detection of granulosa cell apoptosis by flow cytometry of Annexin V/IP staining in control (left), FSH only (middle), and FSH+AKT in (right) groups. Cells in lower right quadrant (Annexin V+/PI−) and upper right quadrant (Annexin V+/PI+) were considered to apoptotic cells. (B) The percentages of apoptotic granulosa cells determined by flow cytometry of Annexin V/IP staining. (C) The percentages of dead granulosa cells determined by Trypan blue staining. The bar graphs represent the mean (±S.E.M.) of results from three independent experiments. *Significant differences (P<0.05) when compared with control; †represent significant difference (P<0.05) between them. AKT in, AKT inhibitor.


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