ERK1/2 is involved in luteal cell autophagy regulation during corpus luteum regression via an mTOR-independent pathway

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Abstract: Autophagy is known to be regulated by the phosphoinositide-3 kinase (PI3K)-protein kinase B (AKT) and/or mitogen-activated protein kinase 1/2 (MEK1/2)-extracellular signal-regulated kinase 1/2 (ERK1/2) pathways, leading to activation of mammalian target of rapamycin (mTOR), a major negative regulator of autophagy. However, some reports have also suggested that autophagic regulation by the PI3K-AKT and/or MEK1/2-ERK1/2 pathways may not be mediated by mTOR activity, and there is no direct evidence of the involvement of these pathways in luteal cell autophagy regulation. To elucidate the luteal cell-specific regulatory mechanisms of autophagy induction during corpus luteum (CL) regression, we evaluated whether luteal cell autophagy is regulated by the PI3K-AKT pathway and/or MEK1/2-ERK1/2 pathway and if this regulation is mediated by mTOR. We found that autophagy induction increased despite mTOR activation in luteal cells cultured with prostaglandin F2α (PGF2α), an important mediator of CL regression, suggesting that PGF2α-induced autophagy is independent of mTOR regulation. We also found that PGF2α-induced autophagy was not mediated by AKT activity, because AKT inhibition using a PI3K inhibitor (wortmannin) did not change autophagy induction or mTOR activity. In contrast, ERK1/2 activity increased in PGF2α-treated luteal cells, as did the levels of autophagy induction despite increased mTOR activity. Furthermore, PGF2α-mediated up-regulation of luteal cell autophagy was reversed by addition of ERK1/2 inhibitors, despite a decrease in mTOR activity. These in vitro results suggest that luteal cell autophagy is induced by increased ERK1/2 activity during CL regression, and is independent of mTOR activity. This finding was further supported by in vivo experiments in a pseudo-pregnant rat model, which showed that induction of luteal cell autophagy during CL regression which is not associated with mTOR regulation.

Key words: autophagy / luteal cell / mTOR / AKT / ERK1/2

Introduction

Regression of the corpus luteum (CL), termed luteolysis, is critical for initiation of the next estrous/menstrual cycle and maintenance of ovarian tissue homeostasis (Davis and Rueda 2002; Berisha and Schams, 2005; Stocco et al., 2007). The major activity that causes CL regression is thought to be luteal cell death by apoptosis, a form of programmed cell death (Rueda et al., 1995; Shikone et al., 1996; Telleria et al., 2001). However, apoptosis may not be the only mechanism for luteal cell death in CL regression, because the vast majority of luteal cells has shown evidence of elimination by non-apoptotic mechanisms, such as autophagy and necrosis, during natural or induced CL regression in marmoset monkeys (Fraser et al., 1995, 1999) and humans (Del Canto et al., 2007). We recently reported that autophagy is induced mainly in steroidogenic luteal cells during CL regression and that it promotes their apoptotic cell death their via an increase in the BAX:BCL2 ratio followed by caspase activation (Choi et al., 2011). These results suggest that autophagy is directly involved in CL regression and plays an important role in the regulation of luteal cell apoptosis. For these reasons, analyses of the molecular events that occur during luteal cell death are pivotal to our understanding of how this process is controlled during CL regression. However, the molecular pathways that regulate autophagy induction in luteal cells during CL development and regression have not yet been elucidated.

In many normal and cancer cells, the mammalian target of rapamycin (mTOR) has been found to be a major negative regulator of autophagy.
The activity of mTOR is regulated primarily by signals such as those provided by the class I phosphoinositide-3 kinase (PI3K)-protein kinase B (AKT) pathway (Corcelle et al., 2009; Chen and Karantza, 2011). Indeed, in various cell types, up-regulation of AKT activity via increased activity of the PI3K-AKT pathway results in phosphorylation of the mTOR repressor, tuberous sclerosis complex 2 (TSC2), 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. Similarly, the mitogen-activated protein kinase 1/2 (MEK1/2)-extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway activates mTOR by phosphorylating TSC2 (Roux et al., 2004; Ma et al., 2005; Stevens et al., 2009), which suggests that enhanced ERK1/2 activity may also inhibit autophagy through activation of mTOR. Therefore, autophagy appears to be regulated by a cellular network that involves upstream signaling pathways integrated by mTOR.

However, recent studies have shown that autphagic regulation via the PI3K-AKT pathway may not be mediated by suppression of mTOR activity, because down-regulation of AKT activity promoted autophagy induction without inhibition of mTOR in HL-60 myeloblastic cells (Saeki et al., 2003) and human colon cancer cells (Ellington et al., 2006). Furthermore, up-regulation of ERK1/2 activity via enhanced activity of the MEK1/2-ERK1/2 pathway was shown to promote autophagy induction in an mTOR-independent manner, potentially through controlling isolation membrane flux, by initiating events that ultimately inhibited a G-protein-coupled receptor associated with the endoplasmic reticulum and Golgi apparatus (Ogier-Denis et al., 2011), leading to decreased autophagy activity of the MEK1/2-ERK1/2 pathway was shown to promote autophagy induction in an mTOR-independent manner, potentially through controlling isolation membrane flux, by initiating events that ultimately inhibited a G-protein-coupled receptor associated with the endoplasmic reticulum and Golgi apparatus (Ogier-Denis et al., 2011).

In the present study, we therefore evaluated the involvement of the PI3K-AKT pathway, the MEK1/2-ERK1/2 pathway and mTOR activity in luteal cell autophagy to elucidate the molecular pathways regulating autophagy induction of luteal cells during CL regression.

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 (26-day-old) female Sprague-Dawley rats were superovulated by i.p. injection of 50 IU pregnant mare serum gonadotropin (PMSG; Sigma Chemical Co., St. Louis, MO). After 48–56 h, rats were injected with human chorionic gonadotropin (hCG; 50 IU i.p., Sigma) to induce ovulation and pseudopregnancy. Rats were killed by cervical dislocation and ovaries were excised 1, 7, 14 and 20 days after hCG administration. These time points represent early-luteal stage (Day 2), mid-luteal stage (Day 7) and late-luteal stage (Days 14 and 20).

Luteal cell collection and in vitro culture

Ovaries were excised 2, 7, 14 and 20 days after hCG injection and placed in Dulbecco’s modified Eagle’s/F12 medium (DMEM/F12, Gibco BRL, Grand Island, NY, USA) that was supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 10 mg/ml streptomycin sulfate (Sigma) and 75 mg/ml penicillin G (Sigma). CLs were separated from ovaries under a dissecting microscope. Isolated CLs were suspended in the appropriate solution for immunoblotting or used for luteal cell collection. For in vitro experiments, luteal cells were isolated from rat ovaries on Day 7 of pseudopregnancy and cultured according to the method described by Behrman et al. (1980) with minor modifications. Briefly, CLs were digested with collagenase-DNase (Sigma). The contents of the flask were filtered through a 70-mm Falcon nylon mesh (Becton Dickinson, Franklin Lakes, NJ, USA) to remove debris, and the filtrate was centrifuged at 200g for 6 min to sediment the suspended cells. Cell pellets were washed twice with Dulbecco’s modified Eagle’s medium (DMEM—F12 (Gibco BRL) supplemented with 10% FBS (Gibco BRL)). Preparations were enriched in luteal cells by centrifugation (200g for 30 min) through a 40% Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient in 0.9% saline. The collected cells were >90% viable, as determined by exclusion of Trypan blue dye. In each experiment, luteal cells were pooled from several animals. Collected cells were seeded at a concentration of 1 x 10⁶ cells/ml in culture dishes made of polyl-l-lysine-coated non-fluorescent thin-bottom glass (MatTek, Ashland, MA, USA). The cells were incubated at 37°C in 5% CO₂ in DMEM—F12 supplemented with 10% FBS, glutamine, HEPES and 100 U/ml penicillin/ml and 100 mg/ml streptomycin (Gibco BRL). Previous studies showed that under these conditions, cells attach, are stereodriogenic and remain viable. After 72 h of incubation, the medium and unattached cells were removed and replaced with serum-free Earle’s Balanced Salt Solution (EBSS) medium (Sigma) for serum starvation. To induce luteal regression, luteal cells were also cultured in EBSS medium with prostaglandin F2α (PGF2α; 1 µM, Cayman Chemical Company, Ann Arbor, MI, USA) for 15, 30 and 60 min. In addition, luteal cells were cultured in EBSS medium with added PI3K inhibitor (100 nM, wortmannin, Sigma) for 30 min to inhibit the activity of the PI3K-AKT pathway. Finally, luteal cells were cultured in EBSS medium supplemented with PGF2α (1 µM) and specific inhibitors of ERK1/2, U0126 (30 µM, Sigma) or PD98059 (30 µM, Sigma) for 30 min to assess PGF2α-stimulated ERK activity. Treatments were stopped by removing the media, and luteal cells were scraped to extract proteins or fixed for immunofluorescence and electron microscopy analysis. Autophagy of luteal cells was evaluated using acridine orange staining.

Western blot analysis

During autophagy, 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 measured the expression level of LC3-II protein by western blot analysis to evaluate luteal cell autophagy. AKT and ERK1/2 activity were determined by measuring the phosphorylated active forms of AKT and ERK1/2, respectively. mTOR activity was also evaluated by analyzing the phosphorylation of its substrate, ribosomal protein S6 kinase (S6K) (Sarbassov et al., 2005). Freshly isolated and cultured luteal cells were lysed with ice-cold radioimmunoprecipitation assay buffer that was supplemented with a protease inhibitor cocktail (Sigma). Cell lysates were incubated on ice for 30 min to completely solubilize cellular proteins, followed by centrifugation (13 000g, 4°C, 30 min). Whole-cell lysates (20 µg/lane) were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Richmond, CA, USA). After blocking nonspecific binding sites with 5% skim milk, membranes were treated with rabbit polyclonal antibodies to LC3 (diluted 1:1000; Cell Signaling Technology, Boston, MA, USA), total or phosphorylated AKT (Ser473) (diluted 1:1000 and 1:1000; Cell Signaling Technology, respectively), total or
Transmission electron microscopy analysis

To identify autophagic vacuoles at the ultrastructural level, luteal 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, post-fixed in 1% OsO4 in cacodylate buffer, dehydrated and embedded in Epon. Ultrathin sections were counterstained with hematoxylin, dehydrated and mounted.

Immunofluorescence staining

Luteal cells were cultured on sterilized glass coverslips, fixed with 4% paraformaldehyde and blocked with 0.1% bovine serum albumin (BSA) in PBS. Cells were incubated with anti-LC3 rabbit polyclonal antibody [diluted 1:50] or phosphorylated ERK1/2 mouse polyclonal antibody [diluted 1:1000] 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).

Immunohistochemistry

Paraffin-embedded whole ovarian sections were deparaffinized, 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. Non-specific binding was blocked with 5% BSA (Sigma) in PBS for 30 min. After washing, sections were incubated overnight at 4°C with anti-30 min. Peroxidase activity was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Integrated optical intensities of the immunoreactive bands were quantified by imaging (Gel Doc 2000, Bio-Rad) and the analysis software Quantity One ver. 4.0.3 (Bio-Rad). Expression of LC3-II was normalized to β-actin, while expression levels of phosphorylated AKT, ERK1/2 and S6K were normalized to those of total AKT, ERK1/2 and S6K, respectively.

Detection and quantification of acidic vesicular organelles with acridine orange

Autophagy is the process of sequestering cytoplasmic proteins into the lytic component and is characterized by the formation of numerous acidic vesicles that are referred to as acidic vesicular organelles (AVOs) (Bursch et al., 2000; Paglin et al., 2001). To detect and quantify AVOs in luteal cells, we performed vital staining with acridine orange. In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, respectively, whereas acidic compartments fluoresce bright red, as described previously (Traganos and Darzynkiewicz, 1994; Stankiewicz et al., 1996). The intensity of red fluorescence is proportional to the degree of acidity and/or the volume of the cellular acidic compartment. This allows measurement of a change in the degree of acidity and/or the fractional volume of the cellular acidic compartment. Luteal cells were stained with acridine orange at a final concentration of 1 μg/ml for a period of 15 min, removed from the plate with trypsin-EDTA and collected in phenol red-free growth medium. Green (510–530 nm) and red (650 nm) fluorescence emission from 10,000 cells illuminated with blue (488 nm) excitation light was measured with an FACSaria flow cytometer (BD Biosciences, Heidelberg, Germany). Data analysis was conducted using the CellQuest software.

Statistical analysis

Expression levels of all proteins and proportions of autophagic cells are reported as means ± standard errors. Statistical analysis was performed using ANOVA. Significant differences between treatment groups were determined by Duncan’s multiple-range test. Statistical significance was inferred at P < 0.05.

Results

Effects of prostaglandin F2α on autophagy induction and its regulation in luteal cells

To determine the effect of prostaglandin F2α (PGF2α) on autophagy induction, mTOR activity, and the PI3K-AKT and MEK1/2-ERK1/2 signaling pathways, luteal cells were cultured in serum-free medium with PGF2α (100 mM) for 60 min, and the expression of LC-III and phosphorylation of S6K, AKT and ERK1/2 were evaluated. As shown in Fig. 1A and B, PGF2α treatment significantly increased LC-III expression at 30 and 60 min. Similarly, phosphorylation of S6K and ERK1/2 increased significantly in response to PGF2α treatment (Fig. 1B; P < 0.05). In contrast, phosphorylation of AKT decreased significantly in response to PGF2α treatment (Fig. 1B; P < 0.05).

PGF2α-mediated autophagy induction and mTOR activation are not under the influence of AKT

We examined the effect of AKT inhibition on the level of phosphorylated S6K and LC-III expression to determine whether inhibition of AKT activity affects mTOR activity and autophagy induction in luteal cells. As shown in Fig. 2A and B, PGF2α treatment decreased AKT activity significantly, which was accompanied by an increase in S6K phosphorylation and LC-III expression (P < 0.05). Although treatment of cells with wortmannin (PI3K inhibitor) also decreased AKT phosphorylation significantly (P < 0.05), S6K phosphorylation and LC-III expression did not change in wortmannin-treated luteal cells.

PGF2α treatment induces luteal cell autophagy through ERK1/2 activation

We examined the effect of ERK1/2 inhibition on the level of phosphorylated S6K and LC-III expression to determine whether increased ERK1/2 activation promotes mTOR activity followed by autophagy induction in luteal cells cultured with PGF2α. As shown in Fig. 3A and B, the effect of PGF2α on ERK1/2 was abolished by addition of two ERK1/2 inhibitors, U0126 and PD98059, and this was accompanied by decreases in S6K phosphorylation and LC-III expression (P < 0.05). To confirm this finding, we examined the endogenous expression of phosphorylated ERK1/2 and the subcellular localization of endogenous LC3 using...
immunofluorescence staining. Phosphorylated ERK1/2 and endogenous LC3 were easily detected as green and red fluorescence, respectively, in cultured luteal cells (Fig. 3C). In luteal cells cultured with PGF2α, phosphorylated ERK1/2 staining increased (Fig. 3C, p-ERK1/2, middle), and more punctuated LC3-II structures accumulated in the cytoplasm (Fig. 3C, LC3, middle). In contrast, addition of U0126 to PGF2α-treated cells resulted in weak phosphorylated ERK1/2 immunoreactivity (Fig. 3C, p-ERK1/2, right) and few punctuate LC3-II structures throughout the cytoplasm (Fig. 3C, LC3, bottom). Subsequently, we also took transmission electron microscopy (TEM) images of luteal cells cultured in medium with PGF2α with or without U0126 (Fig. 3D). Autophagic structures are characterized by the presence of multiple autophagosomes, which are double-membranous vacuoles containing engulfed cytoplasmic materials. Autophagosomes increased markedly in luteal cells cultured with PGF2α (Fig. 3D, middle) compared with control cells (Fig. 3D, left). When luteal cells were cultured with PGF2α and U0126, however, the number of autophagosomes decreased compared with that in luteal cells cultured with PGF2α alone (Fig. 3D, right). In

Figure 1 Expression of LC3-II and phosphorylation of S6K, AKT and ERK1/2 in cultured rat luteal cells after incubation under serum-free conditions in the presence of PGF2α. (A) Representative immunoblot of LC3, S6K, AKT and ERK1/2 protein content in luteal cells cultured with PGF2α. (B) Densitometric quantification of LC3-II, phosphorylated S6K (p-S6K), p-AKT and p-ERK1/2 protein content. Experiments were repeated three times and data are expressed as the mean ± SE. *Significant differences (P < 0.05) when compared with 0 min.

Figure 2 Effects of AKT inhibition on S6K phosphorylation and autophagy induction in rat luteal cells cultured with PGF2α. (A) Representative immunoblot of AKT, S6K and LC3 proteins in luteal cells cultured in the absence or presence of PGF2α and AKT inhibitor. (B) Densitometric quantification of phosphorylated AKT (p-AKT), p-S6K and LC-II proteins. Experiments were repeated three times, and data are expressed as the mean ± SE. *Significant difference (P < 0.05) when compared with control.
addition, flow cytometry assays using acridine orange staining revealed that the proportion of autophagic cells was greater among PGF2α-treated luteal cells (19.2-fold greater) than among luteal cells cultured in the absence of PGF2α (control) (Fig. 3E and F). In contrast, PGF2α-induced autophagy decreased significantly, by ~54%, after the addition of U0126 ($P < 0.05$).

**Figure 3** Effects of ERK1/2 inhibition on S6K phosphorylation and autophagy induction in rat luteal cells cultured with PGF2α. (A) Representative immunoblot of ERK1/2, AKT, S6K and LC3 proteins in luteal cells cultured with PGF2α, U1025 and/or PD98059. (B) Densitometric quantification of phosphorylated ERK1/2 (p-ERK1/2), p-S6K and LC-II proteins. Experiments were repeated three times, and data are expressed as means ± SE. *Significant differences ($P < 0.05$) when compared with control; a and b represent significant differences ($P < 0.05$). (C) Double-immunofluorescence staining for p-ERK1/2 and LC3 in luteal cells. p-ERK1/2 and LC3 are stained with red and green fluorescence, respectively. LC3-I had a diffuse distribution within the cytoplasm, whereas LC3-II was present in punctuate structures. (D) Transmission electron microscopic images of luteal cells cultured without PGF2α (control, left), with PGF2α alone (middle) or with PGF2α and U0126 (right). Arrows indicate representative autophagosomes. (E) Representative images of flow cytometry of acridine orange (AO) staining in control (left), PGF2α alone (middle) and PGF2α and ERK1/2 inhibitor (right) groups. FL1-H indicates green color intensity, while FL3-H indicates red color intensity. Cells in the upper left and right quadrants (AO red positive) were considered to be autophagic cells. (F) Percentages of autophagic luteal cells determined by flow cytometry of acridine orange-stained cells.

Luteal cell autophagy induction and phosphorylation of mTOR and ERK1/2 during pseudopregnancy

We used an established in vivo pseudo-pregnant rat model to induce CL development and regression to determine the involvement of ERK1/2.
Regulation of luteal cell autophagy

**Figure 4** Expression of LC3-II and phosphorylation of S6K, AKT and ERK1/2 in luteal cells from pseudo-pregnant rats at different time points (2, 7, 14 and 20 days) after pseudopregnancy. (A) Representative immunoblot of LC3, S6K, AKT and ERK1/2 proteins in luteal cells from pseudo-pregnant rats. (B) Densitometric quantification of LC3-II, phosphorylated S6K (p-S6K), p-AKT and p-ERK1/2 proteins. Experiments were repeated three times and data are expressed as means ± SE. *Significant differences (P < 0.05) when compared with Day 0.

and mTOR. As shown in Fig. 4A and B, L3-II expression on Days 7, 14 and 20 was significantly higher (1.15-, 1.79- and 2.66-fold greater, respectively) than on Day 2 of pseudopregnancy (P < 0.05). Similarly, the phosphorylation of ERK1/2 and S6K increased significantly during pseudopregnancy, and reached a maximum on Day 20 of pseudopregnancy (P < 0.05; Fig. 2B).

We also examined the co-localization of β-honest significant difference, LC3, phosphorylated S6K and phosphorylated ERK1/2 in pseudopregnant rat ovaries. Based on β-honest significant difference expression in steroidogenic luteal cells, CLs were classified as healthy or regressing CLs. A healthy CL was defined as one with intense β-honest significant difference staining of luteal cells on Days 2 (early-) and 7 (mid-luteal phase) of pseudopregnancy (Fig. 5: IA and IE), whereas a regressing CL was classified as one with weak β-honest significant difference staining (Fig. 5: IIa and IIe) on Days 14 and 21 of pseudopregnancy (late-luteal phase). Luteal cells of healthy CLs on Days 2 and 7 of pseudopregnancy stained weakly for the LC3 protein (Fig. 5: IB and IF), and also showed weak immunoreactivity for phosphorylated ERK1/2 (Fig. 5: IC and IG) and S6K (Fig. 5: ID and IH). In contrast, luteal cells with intense LC3 immunoreactivity (Fig. 5: IIB and IIIF) stained intensely for phosphorylated ERK1/2 (Fig. 5: IIC and IIIG) and S6K (Fig. 5: IID and IIHI) on Days 14 and 20 of pseudopregnancy.

**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 (Fujihara et al., 2007; Degtyarev et al., 2009). Luteal cell autophagy has been recognized as the cellular mechanism responsible for CL regression by affecting apoptotic cell death (Choi et al., 2011). However, the role and involvement of the PI3K-AKT pathway and mTOR in the regulation of luteal cell autophagy has remained unclear. Here we examined the involvement of the PI3 K/AKT pathway and mTOR in the regulation of luteal cell autophagy during CL regression by in vitro treatment of primary cultures of luteal cells with PGF2α. PGF2α is an important mediator of CL regression, and has been shown to induce functional and structural regression of the CL in most non-primate species (Davis and Rueda, 2002; Stouffer, 2006; Weems et al., 2006). We previously demonstrated up-regulation of autophagy in luteal cells cultured with PGF2α in vitro (Choi et al., 2011). In this study, we confirmed that PGF2α promoted luteal cell autophagy by examining LC3-II expression, but we also found that PGF2α-stimulated S6K phosphorylation in luteal cells, suggesting that PGF2α-induced luteal cell autophagy may not be mediated via suppression of mTOR activity. This finding is consistent with observations that mTOR-independent pathways regulate autophagy in other mammalian cell models, including C2C12 myotubes (Mordier et al., 2000) and human colon cancer cells (Ellington et al., 2006). Specifically, the latter study demonstrated that suppression of AKT activity stimulated autophagy induction through an mTOR-independent pathway. Similarly, we demonstrated that PGF2α-induced autophagy was accompanied by a decrease in AKT phosphorylation. Therefore, PGF2α-induced luteal cell autophagy may also be mediated by AKT inhibition in an mTOR-independent manner. However, LC3-II expression and S6K phosphorylation did not change when luteal cells were cultured with a PI3K inhibitor (wortmannin) to mimic suppression of AKT activity by PGF2α. These findings suggest that the PI3K-AKT pathway is not involved in the regulation of autophagy induction and mTOR activity in response to PGF2α.

Some previous studies reported that up-regulation of ERK1/2 activity via activation of the MEK1/2-ERK1/2 pathway could promote autophagy in an mTOR-independent manner (Ogier-Denis et al., 1995, 2000; Petiot et al., 1999). In these reports, induction of autophagy by serum starvation was correlated with activation of ERK1/2 in cancer cells. Similarly, we showed that luteal cell autophagy induced by PGF2α treatment was associated with ERK1/2 phosphorylation, as the expression patterns of phosphorylated ERK1/2 were similar to the
expression patterns of LC3-II following PGF2α treatment. Furthermore, PGF2α-mediated up-regulation of LC3-II expression was prevented by inhibition of ERK1/2 phosphorylation using two specific inhibitors of ERK1/2 (U1026 or PD98059), thus enhanced ERK1/2 activity appears to be necessary for PGF2α-induced autophagy induction in luteal cells. These results indicate that PGF2α induces autophagy of luteal cells via activation of ERK1/2, suggesting a pro-autophagic role for ERK1/2 during CL regression. This finding is further supported by the observation that the PGF2α-induced increase in the number of autophagosomes and proportion of autophagic cells was reversed by co-treatment with PGF2α and U0126. Therefore, activation of ERK1/2 is a key event in the induction of luteal cell autophagy following PGF2α treatment. In addition, previous studies reported that PGF2α stimulates mTOR activity in steroidogenic luteal cells through increased ERK1/2 activity (Arvisais et al., 2006, 2010). This finding is confirmed by our observation that PGF2α-stimulated ERK1/2 was suppressed by addition of ERK1/2 inhibitors, which was accompanied by a decrease in S6K phosphorylation. However, PGF2α-induced autophagy was prevented despite

**Figure 5** Immunostaining of 3β-honest significant difference, LC3, phosphorylated ERK1/2 (p-ERK1/2) and p-S6K proteins on adjacent sections of healthy (I) or regressing (II) CLs from pseudo-pregnant rats 2–20 days after pseudopregnancy. Bars = 100 mm.
suppression of mTOR activity through ERK1/2 inhibition, suggesting that ERK1/2 regulates luteal cell autophagy via an mTOR-independent pathway. Taken together, our in vitro results suggest that luteal cell autophagy is induced by enhancement of ERK1/2 activity during CL regression and is independent of mTOR activity.

To confirm our in vitro results showing involvement of ERK1/2 in the regulation of luteal cell autophagy during CL regression, we examined autophagy induction and the activity of mTOR and ERK1/2 in pseudo-pregnant rat ovaries, because a pseudo-pregnant CL can be maintained for only a certain number of days after which spontaneous regression occurs (Leng et al., 2001; Chen et al., 2003). Stage-dependent luteal cell autophagy induction in a pseudo-pregnant CL resulted in an increase in autophagy induction during luteal stage progression, and reached a maximum at the end-point of late-luteal stage (Day 20), resulting in spontaneous CL regression. As expected, cyclic changes in ERK1/2 and S6K phosphorylation coincided with changes in LC3-II expression. These results suggest that up-regulation of ERK1/2 activity via activation of MEK1/2-ERK1/2 signaling is a key event in the induction of luteal cell autophagy during CL regression which is independent of mTOR activity. This finding is further supported by colocalization of LC3 and phosphorylated ERK and S6K proteins in pseudo-pregnant rat ovaries, as immunoreactivity for LC3 and phosphorylated ERK1/2 and S6K increased in regressing CLs in luteal cells weakly stained for 3β-honest significant difference, a steroidogenic marker of luteal cells (Payne et al., 1998). Therefore, we hypothesize that increased ERK1/2 activity induces luteal cell autophagy during CL regression in an mTOR-independent manner.

In conclusion, ERK1/2 activity is directly involved in the regulation of luteal cell autophagy during CL regression which is independent of mTOR regulation.

**Authors’ roles**

J.C. designed the study, interpreted the data and drafted the manuscript. M.J. performed all experiments, interpreted the data and provided critical discussion. E.L. was involved in sample recruitment. D.C. made substantial contributions to the study conception and design, interpretation of results and discussion and the critical review and editing of the final version of the manuscript.

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**Conflict of interest**

None declared.

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