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Ovarian steroid dependence of endoplasmic reticulum stress involvement in endometrial cell apoptosis during the human endometrial cycle

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Abstract

Endoplasmic reticulum (ER) stress is a common cellular stress response that enhances apoptosis to trigger cell death. However, recent studies have shown that estrogen suppresses apoptosis by inhibiting ER stress in some cell types, suggesting that ER stress-induced apoptosis is regulated by ovarian steroid hormones. In endometrial cells, ER stress may also be controlled by ovarian steroid hormones and could be involved in apoptosis induction during the menstrual cycle. To test this hypothesis, we elucidate whether ER stress is regulated by ovarian steroid hormones in human endometrial cells and if it is involved in apoptosis induction. Specifically, we sought to determine the effects of estrogen and progesterone on the PERK/eIF2α/ATF4/CHOP pathway, a pro-apoptotic pathway mediated by ER stress. Our results show that ER stress maker GRP78 expression was increased in human endometrial Ishikawa and endometrial stromal cells (ESCs) treated with tunicamycin. Addition of estrogen decreased tunicamycin-induced GRP78 expression. In contrast, progesterone treatment increased GRP78 in estrogen-treated Ishikawa and ESCs, which significantly increased CHOP expression through phosphorylation of eIF2α and upregulation of ATF4. This upregulation was accompanied by an increased apoptosis induction. The progesterone-induced increase in apoptosis was reversed by either mifepristone (progesterone receptor modulator) or salubrinal (ER stress inhibitor). Furthermore, our *in vivo* results also showed that GRP78, CHOP expression and apoptosis were significantly increased in endometrial cells during the secretory phase as well as by *in vitro* treatment with progesterone. In conclusion, our results suggest that estrogen inhibits ER stress in human endometrial cells. This inhibition is reversed by progesterone during the secretory phase, and this is directly involved in apoptosis induction.

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Introduction

The human endometrium is a dynamic tissue that undergoes cyclic changes, including proliferation, differentiation and degeneration. These sequential changes are regulated by ovarian steroid hormones and apoptosis appears to be important in the cyclic remodeling of the endometrium (Hopwood & Levison 1976, Kokawa *et al.* 1996). Evidence suggests that apoptosis is the common pathway for eliminating senescent endometrial cells from the functional layer of the human endometrium during the late secretory and menstrual phases of the cycle (Kokawa *et al.* 1996, Harada *et al.* 2004). Although endoplasmic reticulum (ER) stress is a crucial mediator of apoptosis induction in various cell types (Logue *et al.* 2013, Iurlaro & Muñoz-Pinedo 2016), it is not clear whether ER stress is involved in apoptosis induction in the endometrial cell cycle.

The ER is a membrane-bound organelle essential for the folding of virtually all secreted and membrane proteins in eukaryotic cells. Under physiological and pathological conditions including hypoxia, energy deprivation and oxidative stress, unfolded proteins will accumulate in the ER and disrupt ER homeostasis, leading to ER stress (Xu *et al.* 2005). To cope with ER stress, cells trigger self-adaptive mechanisms collectively termed the unfolded protein response (UPR) (Zang & Kaufman 2006). The UPR is mainly regulated by three ER-located sensors: PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). In response to ER stress, the activation of these sensors alleviates ER stress by translational attenuation to limit further accumulation of misfolded proteins and to upregulate folding machinery by inducing ER chaperone genes and ER-associated degradation to eliminate misfolded proteins in the ER. (Walter & Ron 2011, Hetz 2012, Wang & Kaufman 2012). These processes reduce the accumulation of unfolded proteins and restore ER function to promote cell viability in adverse environments. However, when ER stress is severe or prolonged and the UPR cannot restore homeostasis, ER stress induces apoptosis (Szegezdi *et al.* 2006). One of the mechanisms of ER stress-induced apoptosis involves

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sequential steps to activate the PERK/eukaryotic initiation factor 2 alpha (eIF2α)/activating transcription factor 4 (ATF4) signaling pathway (Lu *et al.* 2004, Vattem & Wek 2004, Harding *et al.* 2012) and induce pro-apoptotic molecules such as CCAAT/enhancer-binding protein homologous protein (CHOP) (Ron & Habener 1992, Ma *et al.* 2002). During prolonged ER stress, PERK activation increases eIF2α phosphorylation and subsequently selective translation of ATF4, which upregulates CHOP. CHOP is involved in ER stress-induced apoptosis, as evidenced by observations that overexpression of CHOP promotes cell cycle arrest and apoptosis but that CHOP deficiency protects cells from ER stress-induced apoptosis (Oyadomari *et al.* 2002). Thus, ER stress is important in apoptosis induction via the PERK/eIF2α/ATF4/CHOP signaling pathway. A study reported that estrogen rescues pancreatic β-cells from high glucose-induced apoptosis via decreased ER stress followed by downregulation of CHOP (Kooptiwut *et al.* 2014). This finding indicates that estradiol can inhibit ER stress-induced apoptosis. Similarly, in osteoblasts, the primary estrogen 17β-estradiol reduces ER stress-induced apoptosis (Guo *et al.* 2014). These findings indicate that ER stress is a signaling component of estrogen action and is an important mediator of the anti-apoptotic function of estrogen. Furthermore, Guzel *et al.* (2011) demonstrated that ER stress in human endometrial cells is suppressed by estrogen. They also found that ER stress is significantly higher in the late secretory phase of the menstrual cycle, which has the lowest estrogen levels. In addition, it is also well known that progesterone, the main hormone during the secretory phase, inhibits estrogen-induced effects such as pro-proliferative and anti-apoptotic effects in the endometrial epithelium (Tabibzadeh 1995, Selam *et al.* 2001). Therefore, we postulated that ER stress may induce endometrial cell apoptosis during the secretory phase of the menstrual cycle and be regulated by ovarian steroids. Although the inhibitory effects of estrogen on ER stress in endometrial cells have been described (Guzel *et al.* 2011), whether progesterone affects ER stress in endometrial cells has not been elucidated. No direct evidence exists about the involvement of ER stress in the PERK/eIF2α/ATF4/CHOP signaling pathway in endometrial cell apoptosis regulation.

We conducted this study to determine whether ER stress is regulated by the ovarian steroids estrogen and progesterone in the endometrial cell cycle and investigated whether ER stress is involved in the induction of endometrial cell apoptosis through the PERK/eIF2α/ATF4/CHOP signaling pathway.

Materials and methods

Tissue collection

For endometrial stromal cell (ESC) cultures, endometrial samples in the proliferative phase were obtained from premenopausal women undergoing hysterectomy for uterine leiomyoma ($n = 4$). No participant had taken oral contraceptives or hormonal agents for at least three months before surgery. Samples were placed in Hank's balanced salt solution and transported to the laboratory for ESC isolation.

For Western blot and immunohistochemical staining, endometrial tissues were obtained at the time of hysterectomy from 18 premenopausal women (average age 42.8 ± 3.5 years) who underwent hysterectomies for benign gynecological reasons (uterine leiomyoma, $n = 18$). Endometrial tissue samples were divided into two categories according to day of menstrual cycle: proliferative (days 1–14, $n = 9$) and secretory phases (days 15–28, $n = 9$). Menstrual cycle day was established based on the patient's menstrual history and was verified by histological examination of the endometrium. Of the 18 samples, paraffin sections of endometrial samples (3 late proliferative, 1 mid and 2 late secretory) from 6 patients were obtained from the Department of Pathology. The remaining samples (2 early and 4 late proliferative, 3 mid and 3 late secretory) were freshly isolated from 12 patients, snap-frozen in liquid nitrogen and stored at −80°C for western blots. This study was approved by the Ethical Committee of Samsung Medical Center. Written informed consent was obtained from all participants.

Chemicals and cell lines

Estrogen, progesterone and mifepristone were from Sigma-Aldrich. Tunicamycin (ER stress inducer) was from Cell Signaling and salubrinal (ER stress inhibitor) was from Selleckchem (Houston, TX, USA). Ishikawa cells, a well-differentiated endometrial adenocarcinoma cell line, were from the American Type Culture Collection.

Human endometrial stromal cell isolation

Proliferative phase ESCs were dissociated and purified from eutopic endometrial tissues using a published procedure (Ryan *et al.* 1994) with minor modifications. Tissues were rinsed with PBS and the endometrial lining dissected from the myometrium, minced and digested with 2 mg/mL type IV collagenase (Sigma) at 37°C for 60 min with agitation. Stromal cells were separated from epithelial glands using 70-μm-pore filters, then 45-μm-pore nylon mesh. Filtered cells were plated in T75 flasks to adhere for approximately 30 min, and flasks were washed with PBS to remove blood cells and debris. Stromal cells were cultured in phenol red-free Dulbecco's modified Eagle's/F12 medium (DMEM/F12; Gibco BRL) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Gibco BRL), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco BRL) in a humidified atmosphere with 5% CO_2 at 37°C until grown to confluence (5–8 days). Medium was changed every other day. At confluence, cells were subcultured in 24-well culture plates using 1 mL medium. Endometrial stromal cell suspension purity was determined by immunostaining with vimentin stromal cell-specific antibodies (Fig. 1A).

In vitro *experiments*

Ishikawa cells and ESCs were seeded at 1×10^6 cells/mL in poly-L-lysine-coated nonfluorescent thin-bottom glass culture dishes (MatTek, Ashland, MA, USA). Cells were incubated

at 37°C in 5% CO_2 in DMEM/F12 supplemented with 10% charcoal-stripped FBS, glutamine, HEPES, 100 U/mL penicillin and 100 mg/mL streptomycin. At 70–80% confluence, cells were cultured in serum-free, phenol red-free DMEM/F12 with tunicamycin (5 µg/mL) alone or tunicamycin (5 µg/mL) + estradiol (10^{-9}, 10^{-8} or 10^{-7} M) for 8 h to determine if estrogen inhibited ER stress in Ishikawa cells and ESCs. To evaluate the effects of progesterone on estrogen-mediated ER stress in Ishikawa cells and ESCs, cells were cultured in serum-free, phenol red-free DMEM/F12 media with estrogen (10^{-8} M) alone or estrogen (10^{-8} M) + progesterone (10^{-7}, 10^{-6} or 10^{-5} M) for 8 h. To study the ER stress-induced PERK/eIF2α/ATF4/CHOP signaling pathway and apoptosis, Ishikawa cells and ESCs were cultured in serum-free, phenol red-free DMEM/F12 medium with estrogen (10^{-8} M) alone and estrogen (10^{-8} M) + progesterone (10^{-6} M) for 24 h. The progesterone receptor modulator mifepristone (2 µM) or the ER stress inhibitor salubrinal (10 µM) was added to the medium 6 h before analysis. Treatments were stopped by removing medium. Cells were harvested by scraping to generate protein extracts or fixed for immunofluorescence. Apoptosis of the Ishikawa and endometrial stromal cells was evaluated using annexin V/propidium iodide (PI) staining.

Western blots

Under ER stress conditions, glucose-regulated protein 78 (GRP78), an ER chaperone, is highly expressed (Kozutsumi *et al.* 1988). Therefore, GRP78 protein was measured by Western blot to evaluate ER stress induction. The activity of the PERK/eIF2α/ATF4/CHOP pathway was examined by quantifying phosphorylated eIF2α, ATF4 and CHOP proteins to determine involved ER stress-induced apoptotic pathways. ER-induced apoptosis via CHOP was determined by measuring the expression level of tribbles homolog 3 (TRIB3), which is known to one of the major mediators involved in ER stress-induced apoptosis via CHOP (Ohoka *et al.* 2005). In addition, apoptosis was also determined by measuring expression of

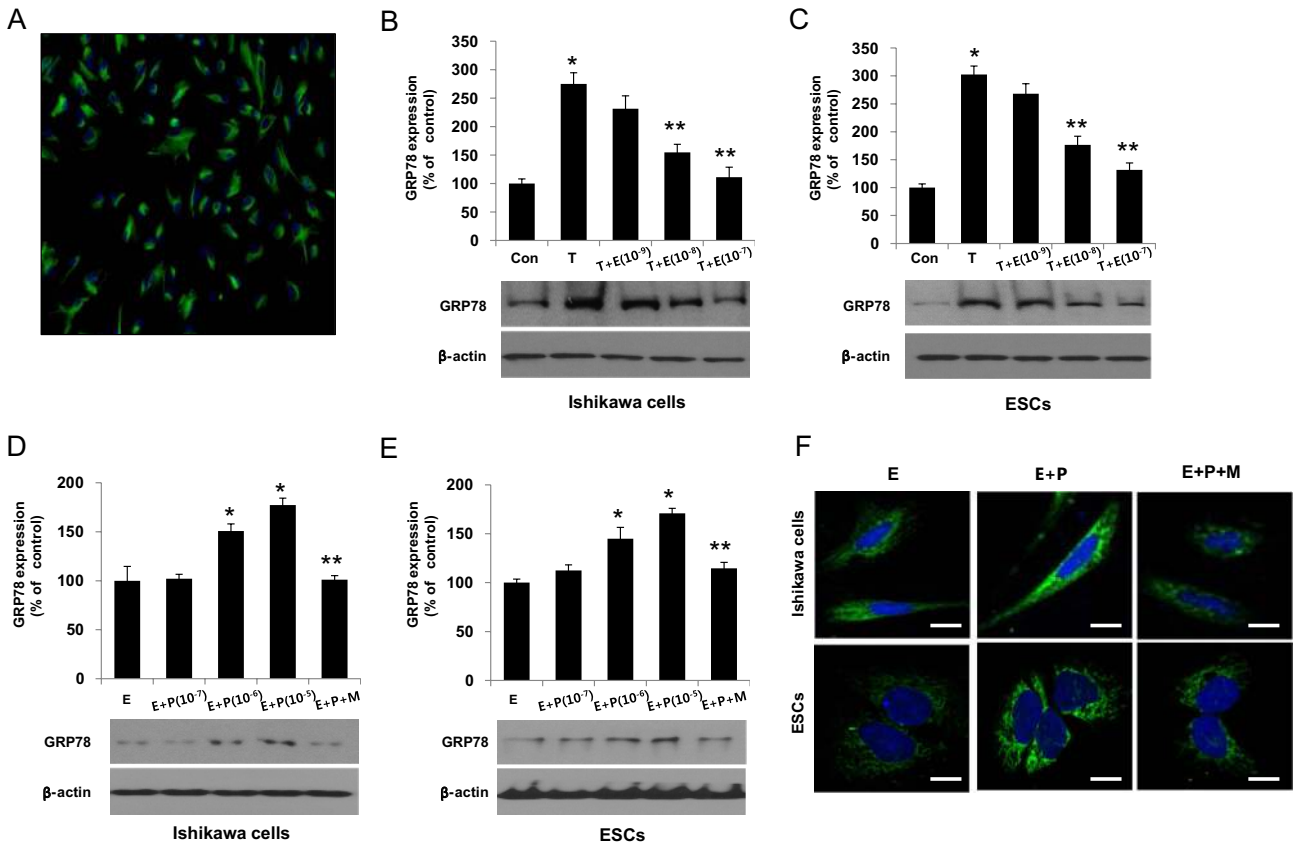

Figure 1 Expression of GRP78 is inhibited by estrogen and enhanced by progesterone in Ishikawa cells and ESCs. (A) Immunofluorescence of vimentin in human endometrial stromal cells. Cells were stained for vimentin protein (green), a stromal biomarker. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). (B and C) Representative immunoblots (top) and densitometric quantification (bottom) of GRP78 in protein extracts from Ishikawa cells (B) and ESCs (C) cultured with tunicamycin after addition of estrogen. Experiments were repeated four times, and data are expressed as percent, where control groups are normalized to 100%. *Significant differences ($P< 0.05$) compared with the tunicamycin-alone group. T, tunicamycin; E, estrogen. (D and E) Representative immunoblots (top) and densitometric quantification (bottom) of GRP78 from Ishikawa cells (D) and ESCs (E) cultured with estrogen after progesterone and/or mifepristone. Experiments were repeated four times. Data are expressed as percent, where cells treated with estrogen alone are normalized to 100%. *Significant differences ($P< 0.05$) compared with the estrogen-alone group. P, progesterone; M, mifepristone. F: Immunofluorescence for GRP78 stained with green fluorophore in cultured Ishikawa cells and ESCs. Bars =10 µm.

BAX, BCL2 and cleaved caspase 3 (Porter & Janicke 1999). Cultured cells and endometrial tissues were lysed with ice-cold radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail (Sigma). For complete solubilization of cellular proteins, lysates were incubated on ice for 30 min and centrifuged at 13,000 ***g*** at 4°C for 30 min. Whole-cell lysates (20 µg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking nonspecific binding sites with 5% skim milk, membranes were incubated with primary antibodies against GRP78 (1:1000, Cell Signaling, #3177), total (1:1000, Cell Signaling, #9722) or phosphorylated eIF2α (Ser473) (1:1000, Cell Signaling, #3597), ATF4 (1:1000, Cell Signaling, #11815), CHOP (1:1000, Cell Signaling, #2895), TRIB3 (1:500, Abcam, #ab137526), BCL2 (1:1000, Cell Signaling, #4223), BAX (1:1000, Cell Signaling, #5023) or cleaved caspase-3 (1:1000, Cell Signaling, #9661) overnight at 4°C. Membranes were washed 3 times for 15 min each with PBS containing 0.1% Tween 20 and incubated with an appropriate secondary antibody IgG (SC-2004 or SC-2005; Santa Cruz Biotechnology) at room temperature for 1 h at a dilution of 1:2000. After three 15-min washes at room temperature, membrane-bound proteins were detected using enhanced chemiluminescence kits (Amersham Pharmacia Biotech). Bands were quantified using NIH ImageJ software (NIH image Processing and Analysis in Java). Expression of GRP78, ATF4, CHOP, BCL2, BAX and cleaved caspase-3 was normalized to *β*-actin; expression of phosphorylated eIF2α was normalized to total eIF2α.

Immunofluorescence

Ishikawa cells and ESCs were cultured on sterilized glass coverslips, fixed with 4% paraformaldehyde and blocked with 0.1% bovine serum albumin in PBS. Cells were incubated with anti-GRP78 rabbit polyclonal antibody (1:50; Abcam; ab32618) in PBS, and then Alexa 488-conjugated secondary antibodies (1:1000; Abcam; ab15007). Slides were mounted in mounting medium (Vector Laboratories) with 4′,6-diamidino-2-phenylindole for nucleus staining. Images were captured with a confocal microscope (Bio-Rad).

siRNA transfection

Cells were transfected according to Invitrogen protocols with some modifications. Cells were seeded 6 well plate and allowed to grow up to 60–80% confluency. Then, cells were washed once with Opti-MEM medium (Invitrogen) and transfected with CHOP siRNA (sc-35437) or nonspecific control siRNA (sc-37007). Briefly, for each well, 9 µL of Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) were diluted with 150 µL of Opti-MEM medium in a tube. In another tube, 3 µL of 10 µM siRNA stock were diluted with 150 µL of Opti-MEM medium. Diluted transfection reagent was gently mixed with diluted siRNA solution and incubated at room temperature for 5 min. This mixed siRNA solution (250 µL) was added to each wells containing cells and incubated at 37°C in 5% CO_2. The final concentration of siRNA was 25 pmol. Next day, cells were treated with tunicamycin (5 µg/mL) for 8 h. Treatments were stopped by removing the medium. Cells were harvested by scraping to generate protein extracts.

Assessment of Ishikawa and endometrial stromal cell apoptosis

Apoptotic cell percentages were determined with annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. After drug treatment, 1×10^5 cells were pelleted, washed with PBS, resuspended in 100 µL binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM potassium chloride, 1 mM MgCl2 and 2 mM calcium chloride) and incubated with 5 µL annexin V and PI for 15 min at room temperature in the dark. Binding buffer (400 µL) was added, and cells were analyzed using a FACSAria flow cytometry (BD Biosciences, Heidelberg, Germany). At least 10,000 cells were analyzed per treatment. Data analysis used CellQuest software.

Immunohistochemistry

Paraffin-embedded endometrial sections were deparaffinized in xylene, rehydrated through graded alcohol and placed in a steamer for 30 min in 10 mM citric buffer for antigen retrieval. Endogenous peroxide was reduced by incubation of sections with 3% H_2O_2 for 30 min. Nonspecific binding was blocked with 5% bovine serum albumin (BSA; Sigma Chemical Co.) in PBS for 30 min. After several washings in phosphate-buffered saline (PBS), sections were incubated overnight at 4°C with polyclonal anti-GRP78 rabbit polyclonal (1:200; Abcam) or CHOP mouse monoclonal (1:50; Abcam), followed by biotinylated secondary antibody (1:2000; DAKO) for 1 h. After incubation with a streptavidin-peroxidase conjugate for 3–5 min at room temperature, antibody complexes were visualized with diaminobenzidine tetrahydrochloride chromogen. Sections were counterstained with hematoxylin, dehydrated through graded alcohol and mounted. For GRP78 and CHOP, negative controls lacking primary antibody were processed on adjacent tissue sections.

Statistical analysis

Expression of proteins and proportions of apoptotic cells are reported as mean ± standard error of four independent experiments. ANOVA and *post hoc* Tukey test for pairwise comparisons were used for statistical analysis for the Western blot and flow cytometry analyses data on the effects of steroid hormones. A Student *t*-test was performed to compare the average between the 2 groups' *in vivo* data. Statistical analyses were executed using SAS version 9.4 (SAS Institute, Cary, NC, USA). Statistical significance was inferred at $P < 0.05$.

Results

Estrogen and progesterone effects on ER stress induction in Ishikawa cells and ESCs in vitro

To investigate the effect of ovarian steroids on ER stress in endometrial cells, we characterized the effects of

estrogen and progesterone on ER stress marker GRP78 expression in cultured Ishikawa cells and ESCs. Addition of increasing concentrations of estrogen significantly decreased GRP78 expression induced by tunicamycin, an ER stress inducer ($P<0.05$, Fig. 1B and C). In contrast, the expression of GRP78 increased significantly with increasing concentrations of progesterone compared with GRP78 levels in cells cultured with estrogen alone ($P<0.05$, Fig. 1D and E). Progesterone-stimulated GRP78 expression was suppressed significantly by the addition of the progesterone receptor modulator mifepristone.

We conducted immunofluorescence to determine endogenous GRP78 expression. Endogenous GRP 78 was readily detected in cultured Ishikawa cells and ESCs (Fig. 1F). In Ishikawa cells and ESCs cultured with estrogen alone, GRP78 staining was weakly detected in the cytoplasm (Fig. 1F: E). The addition of progesterone resulted in intense GRP78 immunoreactivity (Fig. 1F: E + P), and this change was reversed with the addition of mifepristone (Fig. 1F: E + P + M).

Progesterone-induced ER stress increases CHOP by activating PERK-elfFα-ATF4 stress signaling in Ishikawa cells and ESCs

To determine the involvement of progesterone-mediated ER stress in endometrial cell apoptosis, we evaluated the effects of progesterone on CHOP expression and the upstream PERK-elF2α-ATF4 signaling pathway, specifically expression of GRP78, phosphorylated elF2α, ATF4 and CHOP in estrogen-treated Ishikawa cells and ESCs. GRP78, phosphorylated elF2α, ATF4 and CHOP increased significantly in cultured Ishikawa cells and ESCs with the addition of progesterone compared with cells cultured with estrogen alone ($P<0.05$) (Fig. 2A and B). Progesterone-stimulated GRP78 expression was suppressed by the addition of mifepristone ($P<0.05$). Suppression was accompanied by decreased phosphorylated elF2α, ATF4 and CHOP expression ($P<0.05$).

Involvement of CHOP in the regulation of ER stress-induced endometrial cell apoptosis

Given that the activation of PERK-elF2α-ATF4 signaling by progesterone increased CHOP expression, we further investigated whether CHOP is involved in the regulation of ER stress-induced apoptosis in endometrial cells. To do so, tunicamycin-treated Ishikawa cells and ESCs were transfected with CHOP siRNA and nonspecific control siRNA. Transfection with CHOP siRNA led to decrease of CHOP expression in Ishikawa cells and ESCs exposed tunicamycin compared with those transfected with nonspecific control siRNA ($P<0.05$, Fig. 3A and B). After transfection with CHOP siRNA, the expression of TRIB3, a major mediator for ER stress-induced apoptosis via CHOP, was significantly reduced in Ishikawa cells and compared with those transfected with nonspecific control siRNA ($P<0.05$). This reduction was accompanied by decreased BAX and cleaved caspase-3 without changing BCL2 expression ($P<0.05$).

Progesterone-induced CHOP expression increases endometrial cell apoptosis

To determine if progesterone-induced CHOP expression drove apoptosis in endometrial cells, we evaluated the effects of progesterone and salubrinal on expression of TRIB3, BAX, BCL2 and cleaved caspase-3 in estrogen-treated Ishikawa cells and ESCs. Cells treated with progesterone showed significantly higher expression of CHOP and TRIB3 than cells cultured in estrogen alone ($P<0.05$, Fig. 4A and B). The expression levels of BAX and cleaved caspase-3 protein increased significantly without changing BCL2 protein expression when progesterone treatment increased CHOP and TRIB3 expression in estrogen-treated Ishikawa cells and ESCs ($P<0.05$). However, progesterone-mediated upregulation of CHOP, TRIB3, BAX and cleaved caspase-3 expression decreased with the addition of salubrinal ($P<0.05$). We also conducted flow cytometry assays using annexin V and PI to determine the proportion of apoptotic cells. Apoptotic Ishikawa cells and ESCs treated with estrogen alone increased significantly (by 4.91-fold for Ishikawa cells and 2.11-fold for ESCs) in the presence of progesterone ($P<0.05$, Fig. 4C and D). The proportion of apoptotic progesterone-treated cells decreased by 63.6% for Ishikawa cells and by 45.9% for ESCs after addition of salubrinal ($P<0.05$).

ER stress-induced apoptosis increases in normal eutopic endometrium during secretory phase

We investigated ER stress-induced apoptosis during the menstrual cycle in normal eutopic endometrium by comparing the expression of GRP78, CHOP and cleaved caspase-3 in proliferative and secretory eutopic endometrial tissues. GRP78 and CHOP expression increased during the secretory compared to the proliferative phase (Fig. 5A). Cleaved caspase-3 expression also increased significantly during the secretory phase ($P<0.05$).

Figure 5B shows that immunohistochemical staining for GRP78 and CHOP on the adjacent sections of human endometrium during the menstrual cycle. In the proliferative phase, GRP78 (Fig. 5B II and III) and CHOP (Fig. 5B IV and V) staining in glandular cell layers was weak, and stromal cells were negative or very weakly stained. In contrast, the immunoreactivity for GRP78 (Fig. 5B VII and VIII) and CHOP (Fig. 5B IX and X) increased markedly in glandular cell layers during the secretory compared to the proliferative phase, whereas the immunoreactivity for these proteins increased slightly in stromal cells.

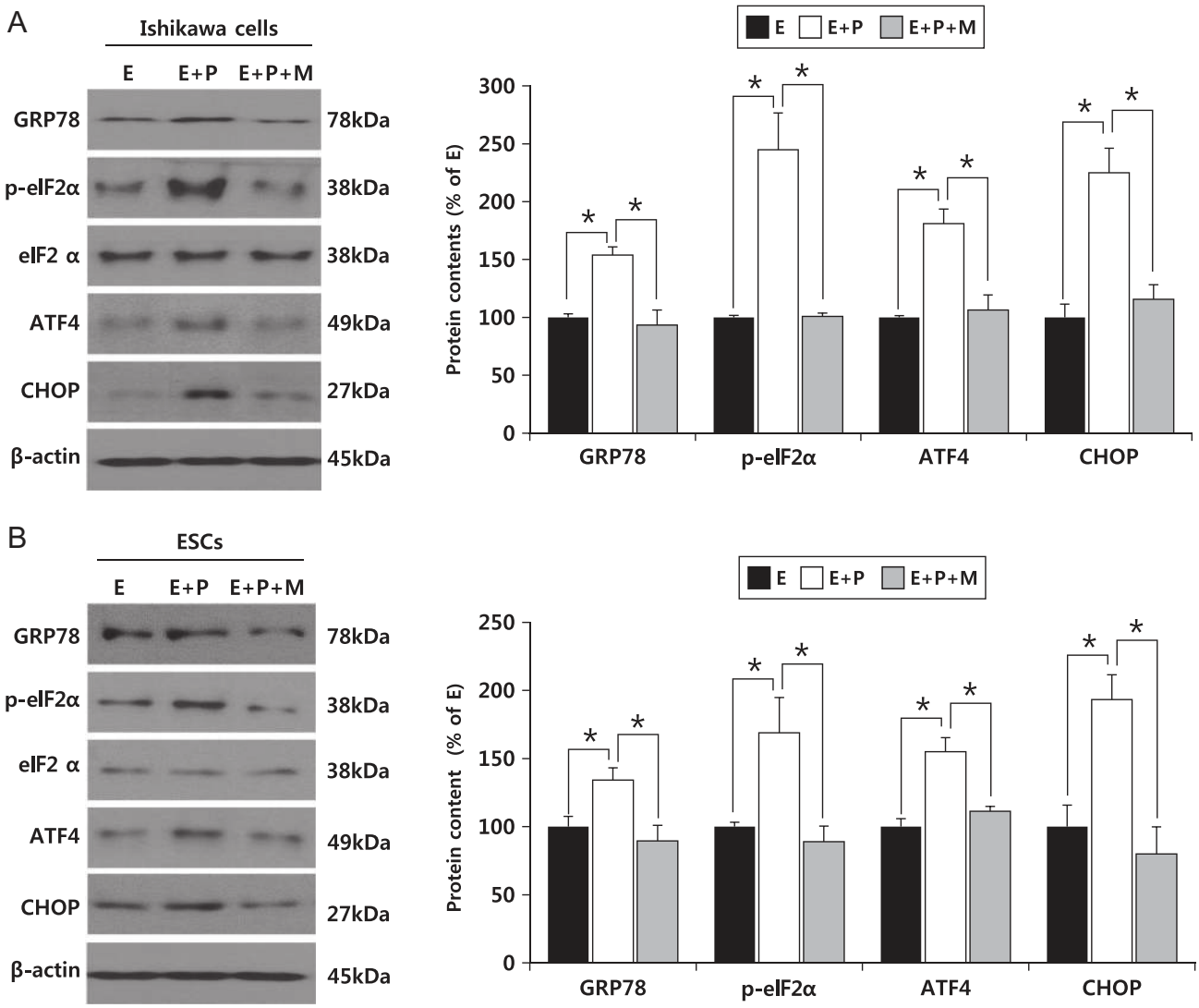

Figure 2 Progesterone-induced ER stress increases CHOP expression by activating the PERK/eIF2α/ATF4 pathway in Ishikawa cells and ESCs. (A and B) Representative immunoblots (left) and densitometric quantification (right) of GRP78, phosphorylated eIF2α, ATF4 and CHOP from cultured Ishikawa cells (A) and ESCs (B). Experiments were repeated four times and data are expressed as percent, where cells treated with estrogen alone are normalized to 100% (*$P<0.05$ by *post hoc* Tukey test). E, estrogen; M, mifepristone; P, progesterone.

Discussion

To the best of our knowledge, this is the first study to reveal that ER stress is enhanced by progesterone and is a crucial mediator of apoptosis induction in endometrial cells during the human endometrial cycle. Although inhibitory of ER stress in human endometrial cells by estrogen was described previously (Guzel *et al.* 2011), studies on the effects of progesterone on ER stress in human endometrial cells and the association between ER stress and apoptosis have not yet been reported. We mimicked ER stress conditions using tunicamycin, a common agent for inducing ER stress (Ozcan *et al.* 2004). Tunicamycin increased the ER stress marker GRP78 in Ishikawa cells and ECSs, whereas addition of estrogen significantly decreased GRP78 expression compared to cells cultured with tunicamycin alone. Thus, the inhibition of ER stress by estrogen was reconfirmed in human endometrial cells. Our study also showed that progesterone significantly increased GRP78 protein in estrogen-treated Ishikawa cells and ESCs, using western blots and immunofluorescence. We found that upregulation of GRP78 by progesterone was suppressed by mifepristone, a potent progesterone receptor modulator. These findings suggested that estrogen suppressed ER stress in endometrial cells, and this was reversed by progesterone. Therefore, progesterone-mediated upregulation of ER stress may promote endometrial cell apoptosis.

To determine whether progesterone-mediated ER stress was associated with endometrial cell apoptosis, we evaluated the effects of progesterone on CHOP,

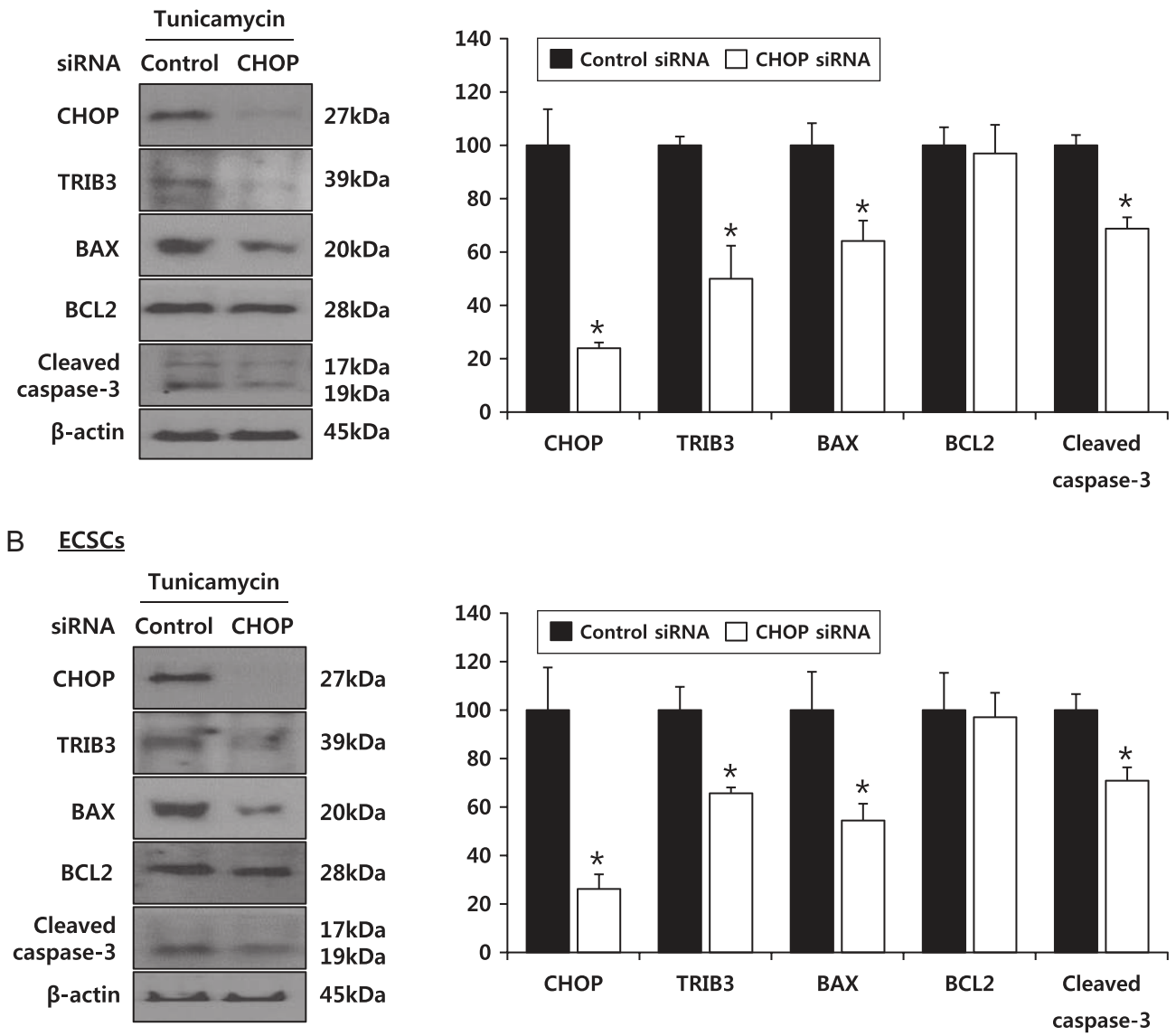

Figure 3 Downregulation of CHOP expression decreases ER stress-induced apoptosis in endometrial cells. (A and B) Representative immunoblots (left) and densitometric quantification (right) of CHOP, TRIB3, BAX, BCL2 and cleaved caspase-3 after the transfection of Ishikawa cells (A) and ESCs (B) with CHOP siRNA and nonspecific control siRNA. Experiments were repeated four times and data are expressed as percent, where cells transfected with and nonspecific control siRNA are normalized to 100%. *$P<0.05$ compared with the control siRNA group.

a critical mediator of ER stress-induced apoptosis (Szegezdi *et al.* 2006). CHOP is expressed at low levels under physiological conditions, but is dramatically upregulated during severe and prolonged ER stress and plays a crucial role in cell arrest and inducing apoptosis (Oyadomari & Mori 2004, Tajiri *et al.* 2004). The PERK/eIF2α/ATF4 signaling pathway has a primary function in inducing CHOP transcription (Fels & Koumenis 2006). When ER stress induces apoptosis, PERK activation leads to phosphorylation of eIF2α, which leads to translational attenuation and induction of ATF4, resulting in CHOP activation, which upregulates pro-apoptotic proteins and downregulates anti-apoptotic proteins to promote apoptosis (Cao *et al.* 2012, Hsu *et al.* 2017). Our studies showed that progesterone-induced ER stress increased CHOP expression by activating PERK/eIF2α/ATF4 signaling, as indicated by increased levels of GRP78, p-eIF2α, ATF4 and CHOP proteins in Ishikawa cells and ESCs. Furthermore, addition of mifepristone attenuated upregulation of GRP78, p-eIF2α, ATF4 and CHOP. These results suggested that progesterone-induced ER stress activated PERK/eIF2α/ATF4 signaling, leading to CHOP

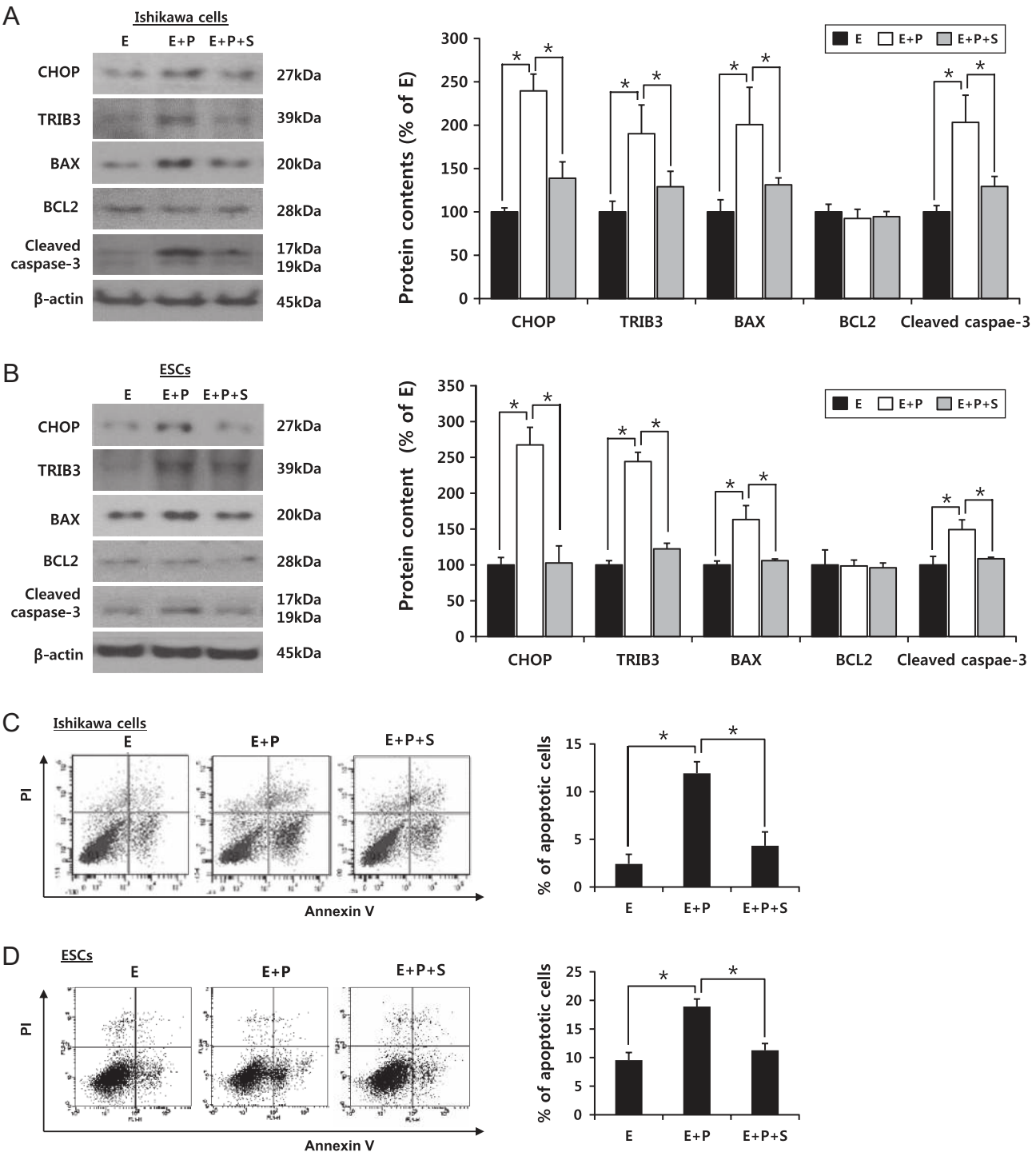

Figure 4 Upregulation of CHOP by progesterone induces endometrial cell apoptosis. (A and B) Representative immunoblots (left) and densitometric quantification (right) of CHOP, TRIB3, BAX, BCL2 and cleaved caspase-3 from cultured Ishikawa cells (A) and ESCs (B). Experiments were repeated four times and data are expressed as percent, where cells treated with estrogen alone are normalized to 100% (*$P<0.05$ by *post hoc* Tukey test). E, estrogen; P, progesterone; S, salubrinal. (C and D) Representative flow cytometry plots (left) and percentages of apoptotic cells from flow cytometry (right) on Ishikawa cells (C) and ESCs (D) treated with estrogen alone, estrogen + progesterone, or estrogen + progesterone + salubrinal. Lower right quadrant, annexin V+/PI−; upper right quadrant, annexin V+/PI+ (apoptotic). Bar graphs display mean ± S.E. (*$P<0.05$ by *post hoc* Tukey test).

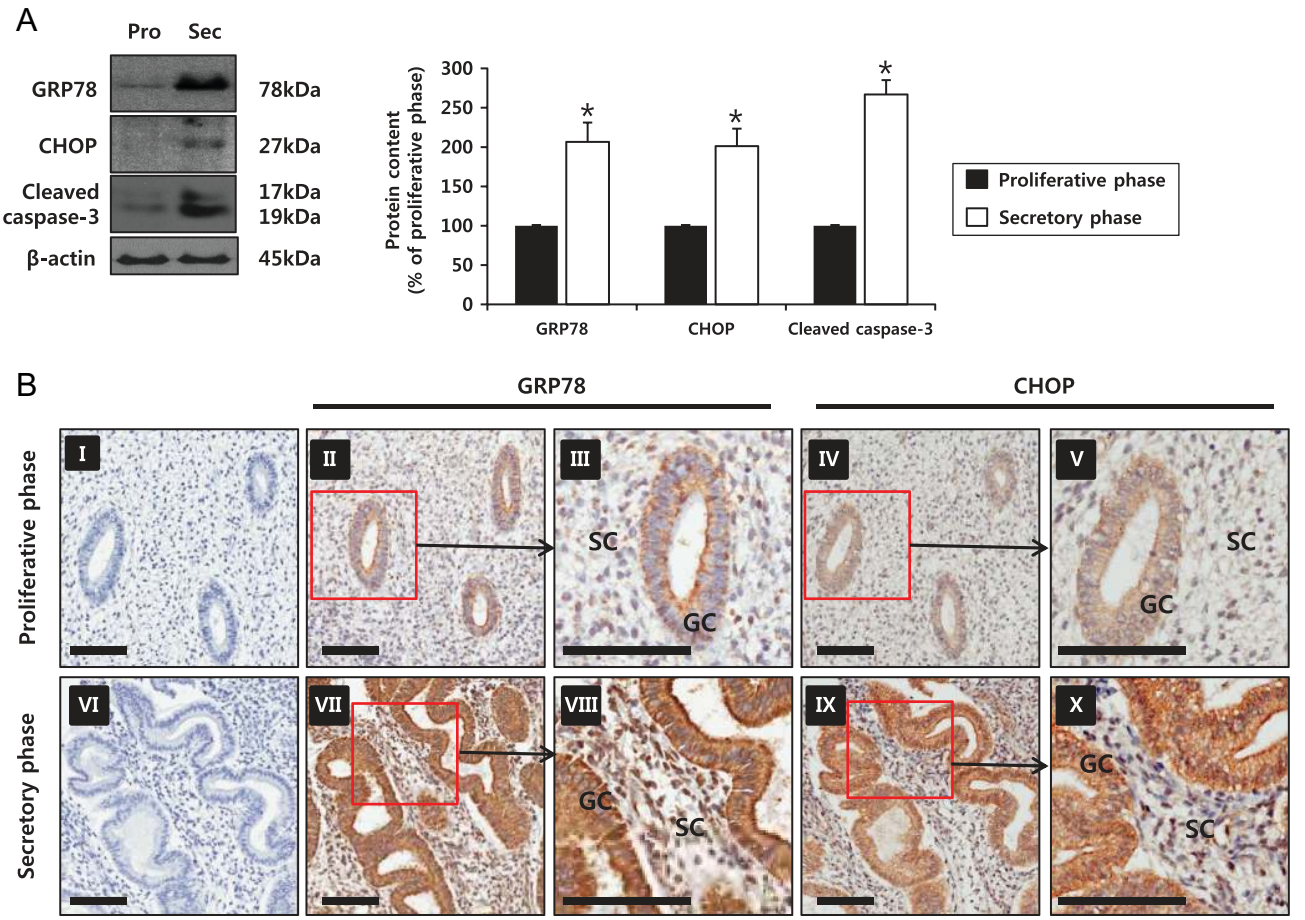

Figure 5 Expression of GRP78, CHOP and cleaved caspase-3 in endometrial tissues during the proliferative and secretory phases of the menstrual cycle. (A) Representative immunoblots (left) and densitometric quantification (right) of GRP78, CHOP and cleaved caspase-3 in endometrial tissues. Pro, proliferative phase; Sec, secretory phase. Experiments were repeated four times and data are expressed as percent, where endometrial tissues in proliferative phase are normalized to 100%. *$P<0.05$ compared with proliferative tissues. (B) Representative micrographs of GRP78 and CHOP immunostaining on adjacent sections of proliferative (I, II, III, IV and V) and secretory endometrium (VI, VII, VIII, IX and X). I and VI are negative controls without primary antibody. II, V, VIII and X represent high magnification of the area enclosed in the red box. GC, endometrial glandular cells; SC, endometrial stromal cells. Bars = 100 μm.

upregulation. Therefore, progesterone-induced ER stress may enhance endometrial cell apoptosis through upregulation of CHOP.

To demonstrate this hypothesis, we evaluated whether CHOP is involved in the regulation of ER stress-induced apoptosis in endometrial cells, using CHOP siRNA study. According to previous studies, upregulation of CHOP expression by ER stress induces apoptosis by increasing TRIB3 expression, which suggests that upregulation of TRIB3 signaling is one of the major mechanisms involved in ER stress-induced apoptosis via CHOP (Ohoka *et al*. 2005, Carracedo *et al*. 2006). In the present study, we found that downregulation of CHOP by siRNA inhibited TRIB3 expression in tunicamycin-treated Ishikawa cells and ESCs, and then decreased BAX expression without changing BCL2 expression. This finding suggests that downregulation of CHOP decreases endometrial cell apoptosis by inhibition of TRIB3 expression as the decreased ratio of BAX (pro-apoptotic protein) to BCL2 (anti-apoptotic protein) is a sign of apoptosis inhibition (Oltvai *et al*. 1993, Williams & Smith 1993). In addition, the active form of caspase 3 (executioner caspase) also decreased by CHOP siRNA in tunicamycin-treated Ishikawa cells and ESCs. Therefore, it is possible that ER stress-mediated CHOP expression is involved in the regulation of endometrial cell apoptosis via TRIB3.

Furthermore, we also found that CHOP combined with TRIB3 expression increased in progesterone-treated Ishikawa cells and ESCs with upregulation of BCL2/BAX ratio and cleaved caspase-3 expression, which suggests that progesterone-induced upregulation of CHOP increased apoptosis induction in endometrial cells. This hypothesis was confirmed by the observation that the progesterone-induced increase in BCL2/BAX ratio and cleaved caspase-3 were prevented when CHOP and

TRIB3 expression was reduced by the ER stress inhibitor salubrinal. This inhibitor targets a specific phosphatase of eIF2α, blocking p-eIF2α dephosphorylation (Boyce *et al*. 2005). Flow cytometry showed that induction of apoptosis coincided with progesterone-induced CHOP expression. Therefore, upregulation of CHOP by progesterone was pivotal for the regulation of ER stress-induced apoptosis by controlling TRIB3 expression in endometrial cells.

These findings were supported by *in vivo* experiments that evaluated if ER stress, CHOP and apoptosis differed in human normal endometrium according to menstrual cycle. GRP78 and CHOP expression were higher in the secretory endometrium than the proliferative phase. Cleaved caspase-3 expression also increased with CHOP expression during the secretory phase. These results indicated that increased ER stress enhanced apoptosis induction through activation of CHOP expression in endometrial cells during the secretory phase of the human endometrial cycle. This finding was supported by immunohistochemical staining for GRP78 and CHOP in the human endometrium, with immunoreactivity for GRP78 and CHOP increasing in glandular cell layers and stromal cells during secretory phase. Furthermore, given the *in vivo* immunohistochemistry results showing obvious GRP78 and CHOP alteration in the glandular cell layers during the secretory phase when serum progesterone level is high, it is more likely that our Ishikawa cell data well reflect endometrial glandular cells, at least in the GRP78 and CHOP expression by progesterone treatment. These results suggested that ER stress increased in the secretory endometrium, and was directly involved in apoptosis induction through regulation of CHOP expression.

In conclusion, ER stress was upregulated by progesterone during the secretory phase in endometrial glandular and stromal sells, promoting endometrial apoptosis via activation of the PERK/eIF2α/ATF4/CHOP signaling pathway. This result provides novel insight into the underlying molecular mechanism of endometrial cell apoptosis by ovarian steroids during the menstrual cycle.

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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