Dienogest enhances autophagy induction in endometriotic cells by impairing activation of AKT, ERK1/2, and mTOR

JongYeob Choi, Ph.D.,[a] MinWha Jo, M.S.,[b] EunYoung Lee, M.S.,[a] Dong-Yun Lee, M.D., Ph.D.,[a] and DooSeok Choi, M.D., Ph.D.[a]

[a] Infertility Clinic, Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine; and [b] Center for Clinical Research, Samsung Biomedical Research Institute, Seoul, South Korea

Objective: To elucidate the therapeutic mechanisms of progestin and the effects of progesterone and progestin (dienogest) on autophagy induction and regulation in endometriotic cells, specifically the effects of progesterone and dienogest on the phosphoinositide-3/protein kinase B (PI3K-AKT) and mitogen-activated protein kinase kinases 1 and 2 (MEK1/2)/extracellular-signal-regulated kinase 1/2 (ERK1/2) pathways, which activate mammalian target of rapamycin (mTOR), a major negative regulator of autophagy.
Design: In vitro study using human endometriotic cyst stromal cells (ECSCs).
Setting: University medical center.
Patient(s): Fifteen patients with ovarian endometrioma.
Intervention(s): ECSCs treated with progesterone or dienogest.
Main Outcome Measure(s): Autophagy as measured by the expression of the microtubule-associated protein light chain 3-II (LC3-II) and autophagosome formation, and levels of AKT, ERK1/2, and mTOR activity to quantify the phosphorylation of AKT, ERK1/2, and S6K (the downstream target of mTOR).
Result(s): Progesterone treatment had not statistically significant effect on LC3-II expression, autophagosome formation, or phosphorylation of AKT, ERK1/2, or S6K in estrogen-treated ECSCs. However, dienogest treatment up-regulated LC3-II expression and stimulated autophagosome formation. These effects were accompanied by decreased activation of AKT, ERK1/2, and S6K. Furthermore, incubation of ECSCs with AKT and ERK1/2 inhibitors, which mimicked dienogest-mediated inhibition of AKT and ERK1/2 activity, suppressed S6K activity, followed by an increase in LC3-II expression. In addition, cotreatment with dienogest and 3-methyladenine (autophagy inhibitor) decreased the levels of apoptosis of ECSCs compared with the single treatment with dienogest.
Conclusion(s): Our results suggest that dienogest treatment of endometriotic cells suppresses AKT and ERK1/2 activity, thereby in turn inhibiting mTOR, inducing autophagy, and promoting apoptosis. (Fertil Steril® 2015;104:655–64. ©2015 by American Society for Reproductive Medicine.)
Key Words: AKT, autophagy, dienogest, endometriosis, ERK1/2

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Endometriosis is defined by the presence of endometrium-like tissue outside the uterine cavity, primarily on the ovaries and pelvic peritoneum. Endometriosis is one of the most common causes of chronic pelvic pain, dysmenorrhea, and infertility (1, 2) and affects about 5% to 15% of all women of reproductive age and 20% to 50% of all infertile women (3, 4). The typical characteristics of endometriosis are increased production of estradiol, which stimulates the proliferation of endometriotic tissue, and perturbations in the progesterone response in a phenomenon known as progesterone resistance (5–7). Therefore, current medical therapies focus either on

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Reprint requests: DooSeok Choi, M.D., Ph.D., Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea (E-mail: dooseok.choi@samsung.com).

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lowering estradiol levels or stimulating the progesterone response (2, 8).

The most effective medical treatment options for endometriosis are gonadotropin-releasing hormone (GnRH) analogues. However, although GnRH agonists provide effective pain relief and suppress the progression of endometriotic implants by inducing hypoestrogenism (9), these treatments are not suitable for long-term applications because of their severe hypoestrogenic effects. On the other hand, progestins, which are synthetic versions of progesterone, reduce serum estrogen levels by preventing ovulation without causing hypoestrogenism (10). Progestins also exert progestogenic effects on estrogen-primed endometrium (11). Therefore, progestin therapy is a practical option for long-term treatment of endometriosis because it is associated with fewer adverse effects. However, the exact mechanism(s) by which progestin acts on endometriotic cells have yet to be elucidated.

Apoptosis is a form of programmed cell death. Accumulating evidence indicates that reduced apoptosis in refluxed endometrial cells might enhance their survival at ectopic sites, thereby promoting the establishment of endometriosis (12, 13). However, apoptosis may not be the only mechanism of endometrial cell death. Autophagy, a nonapoptotic form of programmed cell death, is an intracellular bulk degradation system in which a portion of the cytoplasm is enveloped in double-membrane-bound structures called autophagosomes, which undergo maturation and fusion with lysosomes for degradation (14, 15). It is also known to play an important role in promoting cell death by promoting caspase-dependent apoptosis in some normal (16, 17) and cancer cells (18–20).

We recently showed that the induction of autophagy exerts a proapoptotic effect on normal human endometrial cells (21). However, autophagy is suppressed in endometriotic cells due to derepression of mammalian target of rapamycin (mTOR), a major negative regulator of autophagy, which results in decreased endometriotic cell apoptosis (22). These findings implicate a direct role for mTOR-mediated suppression of autophagy in the pathogenesis of endometriosis.

Previous studies have shown that the class I phosphoinositide-3 kinase (PI3K)/protein kinase B (AKT) and the mitogen-activated protein kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathways negatively regulate autophagy induction by activating mTOR (23, 24), suggesting that inhibition of AKT and ERK1/2 could promote autophagy by preventing mTOR activation. In addition, progestin has been demonstrated to inhibit the PI3K-AKT (25) and MEK1/2-ERK1/2 (26) pathways in endometrial and breast cancer cells, respectively. Therefore, we hypothesized that progestin enhances autophagy induction by inhibiting AKT and ERK1/2, resulting in mTOR inactivation. However, it is not yet known whether progestin regulates AKT and ERK1/2 activity in endometriotic cells, and it is also unknown whether these pathways influence autophagy in endometriotic cells via mTOR signaling. In this study we evaluated the effects of progestin (dienogest) on autophagy in endometriotic cells. Specifically, we determined whether the PI3K-AKT pathway and the MEK1/2-ERK1/2 pathway are involved in progestin-mediated autophagy induction via mTOR signaling.

MATERIALS AND METHODS

Human Endometriotic Cyst Stromal Cell Isolation

Ectopic endometriotic tissue samples (n = 15) were obtained from proliferating ovarian endometriotic cysts (endometrioma). No study participant had taken oral contraceptives or hormone agents for at least 3 months before surgery. The average age of the participants from whom ectopic endometrial tissue was obtained was 29.5 ± 5.4 years. Proliferating endometriotic cyst stromal cells (ECSCs) were dissociated and purified from ovarian endometriotic tissue essentially as described elsewhere (27) but with minor modifications. Briefly, tissue samples were minced with a sterile surgical blade and digested in phosphate-buffered saline (PBS) containing 2 mg/mL of type IV collagenase (Sigma-Aldrich) at 37°C for 60 minutes with agitation. Stromal cells were separated from the epithelial glands by use of 70-μm pore filters followed by 45 μm-pore nylon mesh. Filtered cells were plated in T-75 flasks and allowed to adhere for approximately 30 minutes. The flasks were then washed with PBS to remove blood cells and debris.

The stromal cells were cultured in 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). The cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C, and the medium was changed every other day. Upon reaching confluence, the cells were subcultured in 24-well plates (1 mL/well). Endometrial stromal cell suspension purity was determined by immunostaining with vimentin stromal cell-specific antibodies. This study was approved by the ethics committee of Samsung Medical Center, and written informed consent was obtained from all participants.

In Vitro Experiments

Subcultured ECSCs were seeded at 1×10^6 cells/mL in poly-L-lysine-coated nonfluorescent thin-bottom glass culture dishes (MatTek). The cells were incubated at 37°C in 5% CO_2 in DMEM/F12 supplemented with 10% charcoal-stripped FBS, glutamine, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 U/mL penicillin, and 100 mg/mL streptomycin. Upon reaching 70% to 80% confluence, the cultures were serum starved in serum-free Earle's balanced salt solution (EBSS) medium (Sigma-Aldrich).

To evaluate the effects of progesterone and progestin on autophagy in endometriotic cells, the cells were cultured in EBSS medium before the hormone treatment. After 24 hours of culture, estradiol (10 nM; Sigma-Aldrich) alone, estradiol (10 nM) + progesterone (1 μM; Sigma-Aldrich), or estradiol (10 nM) + dienogest (10 μM; Abcam) were added for 72 hours. In this experiment, we added same amount of dimethyl sulfoxide (DMSO; Sigma-Aldrich) in the culture media of estrogen and/or progesterone-treated groups to minimize