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Taiwanese Journal of Obstetrics & Gynecology 51 (2012) 50–54

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Original Article

Estrogen receptor expression affected by hypoxia inducible factor-1α in stromal cells from patients with endometriosis

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Accepted 7 October 2011

Abstract

Objective: Endometriosis is an estrogen-dependent disease. The aim of this study was to evaluate the different expression of estrogen receptors (ER) and its relation to hypoxia inducible factor-1α (HIF-1α) in stromal cells from women with endometriosis.
Materials and Methods: Paired eutopic endometrial and ectopic endometriotic stromal cells were isolated from women with endometriosis while they underwent laparoscopy. The expression of ERα and ERβ was measured by reverse transcription-polymerase chain reaction and Western blot. Regulation of ER expression was evaluated by HIF-1α knockdown via short interference RNA.
Results: The expression of ERβ was significantly increased in ectopic stromal cells. Treatment of endometrial stromal cells with hypoxia induced ERβ expression. Knockdown of HIF-1α abolished hypoxia-induced ERβ expression and increased ERα expression.
Conclusion: The expression of ERβ is regulated by hypoxia. Results of this study will provide important information in the involvement of hypoxia factors in mediating estrogen action via different ER expression in endometriosis.

Keywords: endometriosis; estrogen receptor α; estrogen receptor β; hypoxia; hypoxia inducible factor-1α

Introduction

Endometriosis is a common gynecologic disease with a complex and multifactorial etiology [1]. It affects about 10% women of reproductive age who usually suffer from symptoms such as dysmenorrhea, dyspareunia, and infertility. Endometriosis is also considered as a highly estrogen-dependent disorder. However, *de novo* synthesis of estrogen by ectopic endometriotic stromal cells can avoid apoptosis and/or maintain the proliferation of these cells even during menstruation when ovarian estrogen supply is limited. Elevated prostaglandin E_2 (PGE_2) induces aberrant steroidogenic acute regulatory protein gene expression, which regulates the first committed step in the biosynthesis of steroids from cholesterol [2]. Activation of the PGE_2 receptor coupled protein kinase A pathway by PGE_2 in endometriotic stromal cells contributes to abnormally local production of estrogen in the development of endometriosis [3].

The biological effects of estrogen involved in cell proliferation, survival/apoptosis, differentiation and development are mediated by estrogen receptor (ER) α and ERβ [4]. ERβ seems to act as a negative modulator of ERα activity. Messenger RNAs (mRNAs) of ERα and ERβ isoforms are expressed in human endometrium and endometriosis, but the results are inconsistent. There is no significant difference between the cycle phases of menstruation or the control and endometriosis groups in these transcripts. However, the eutopic endometrium of healthy women has a higher ERα/ERβ1 ratio than that of endometriotic women during the proliferative phase [5]. In contrast, it has been found that ERβ transcripts in endometriotic stromal cells are significantly increased than stromal cells isolated from endometrium [6].

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1028-4559/$ - see front matter
doi:10.1016/j.tjog.2012.01.010

This ERβ overexpression contributes to cell proliferation and decreases ERα mRNA and protein production in endometriotic stromal cells. The alterations in the relative levels of ERβ and ERα in endometriotic stromal cells affects the estrogen-induced cellular response and may further lead to loss of progesterone signaling and progesterone resistance in endometriosis [4]. Nevertheless, the mechanism responsible for the alteration of ERα/ERβ ratio remains unclear.

In our previous study, hypoxia may play a possible role for survival of retrograded endometrial debris before blood supply is available and for angiogenesis in implanted ectopic endometriotic lesions [7]. Hypoxia inducible factor (HIF)-1α acts as a master transcription regulator of numerous hypoxia-inducible genes under hypoxia condition. For example, HIF-1α-induced leptin accumulation in peritoneal fluid of women with endometriosis contributes to the peritoneal microenvironment of endometriosis through activation of peritoneal macrophages [8] and stimulation of endometriotic stromal cell proliferation [9].

Elevated expression of ERβ is predicted to play a role in the development of endometriosis. However, the underlying mechanism responsible for aberrant expression of ERβ in ectopic endometriotic tissue remains to be clarified. There are possible hypoxia response elements (HREs) on the promoter region of ERs using a bioinformatic method in our preliminary analysis. We hypothesize that increased ERβ expression in endometriotic stromal cells may be regulated by HIF-1α, which may support an etiological insight of hypoxia into the development of endometriosis.

Materials and methods

Patients

The subjects in this study were women of reproductive age who were undergoing laparoscopy at the Department of Obstetrics and Gynecology, National Cheng Kung University Hospital. The classification of endometriosis was determined by the direct vision of laparoscopy according to the revised classification of American Society of Reproductive Medicine [10]. All patients were with normal menstrual cycles, had no endocrine disease and had received no hormone therapy for at least 6 months. This study was approved by the Institutional Review Board of National Cheng Kung University Hospital, and informed consent was obtained from each woman.

Isolation of endometrial and endometriotic stromal cells

Endometrial and endometriotic stromal cells were isolated from tissue as previously described [2]. After purification, stromal cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics in a humidified atmosphere with 5% CO_2 at 37°C. To prevent the contamination of epithelial cells, the isolated stromal cells were immunostained with vimentin (stromal cell-specific) and cytokeratin (epithelial cell-specific) specific antibodies.

Hypoxic treatment

Hypoxic treatment was performed as previously described [11], except that endometriotic stromal cells were cultured in phenol red-free DMEM/F12 medium with 10% FBS.

Reverse transcription-polymerase chain reaction

After hypoxia for 24 hours, total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) according to the standard procedure and 500 ng of the total RNA was subjected to reverse transcription. Target genes were amplified using PCR with different pairs of primers. ERα, forward: 5′-AGGGAGAGG AGTTTGTGTG-3′, reverse: 5′-CAAGGAATGCGATGAAG TAG-3′; ERβ, forward: 5′-GGGCACCTTTCTCCTTTAG-3′, reverse: 5′-CTTCTCTGTCTCCGCACAA-3′; HIF-1α, forward: 5′-TGGACTCTGATCATCTGACC-3′, reverse: 5′-CTCAAGT TG CTGGTCATCAG-3′. The sequence of pyruvate dehydrogenase kinase isozyme 1 (PDK-1) and 18S ribosomal RNA primers were reported previously [11].

Western blotting

Western blotting was performed as previously described [11]. Specific antibodies used in this study included anti-ERα antibody (Upstate, Lake Placid, NY, catalog no: 05-820), anti-ERβ antibody (Upstate, 05-824), and anti-HIF-1α antibody (Novus Biologicals, Littleton, CO, catalog no: NB100-449).

Knockdown of HIF-1α

Short interference RNA (siRNA) against HIF-1α (CCAU-GAGGAAAUGAGAGAAAUGCUU) and GC content-matched scramble control (Invitrogen) were transfected into eutopic stromal cells by lipofectamine 2000 (Invitrogen) according to the standard procedure. After transfection, cells were incubated under normoxia or hypoxia for another 24 hours. Then cells were subjected to reverse transcription-polymerase chain reaction (RT-PCR).

Results

Expression of ERs in stromal cells of women with endometriosis

We first accessed the expression of ERs in paired eutopic and ectopic stromal cells from women with endometriosis. In Fig. 1, the mRNA levels of ERα were slightly decreased in three of four ectopic endometriotic stromal cells, while mRNA levels of ERβ were significantly increased in all ectopic stromal cells when compared to eutopic endometrial stromal cells. Moreover, the ratio of ERα and ERβ mRNA level was decreased in ectopic endometriotic stromal cells (Fig. 1C). Similarly, the protein levels of ERα were slightly decreased, while the protein levels of ERβ were significantly increased in ectopic endometriotic stromal cells when compared to eutopic endometrial stromal cells (Figs. 2A and 2B). The ratio of ERα

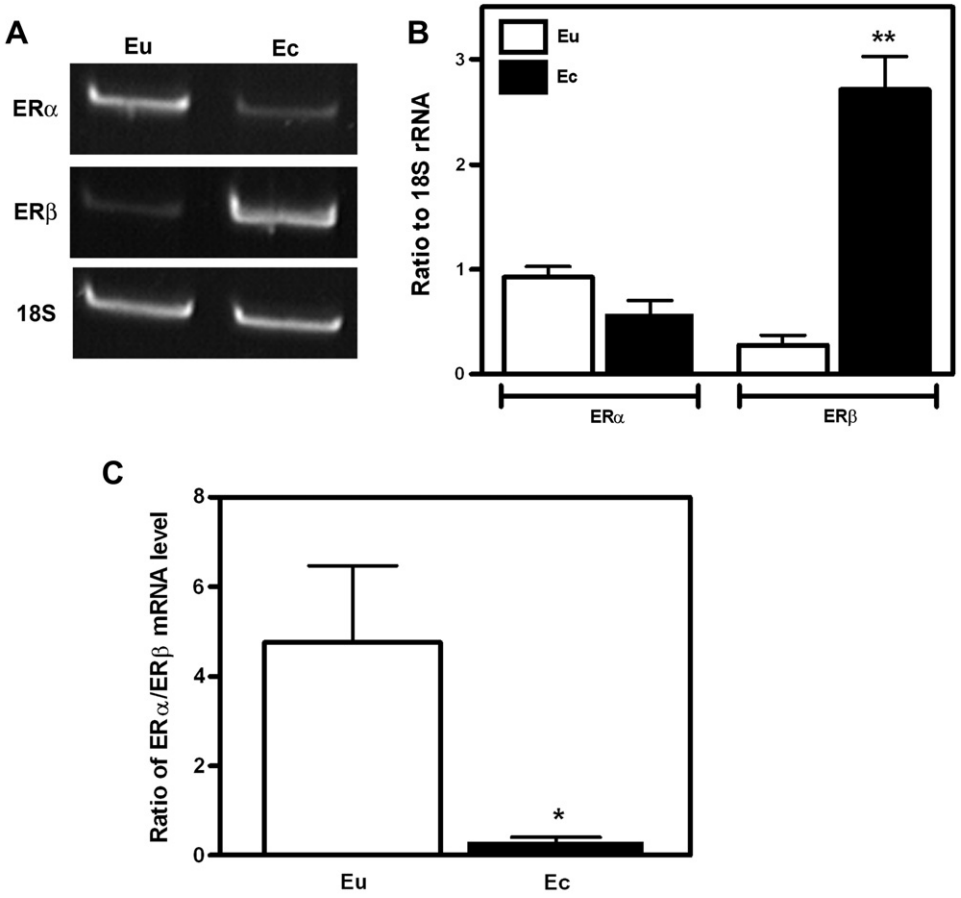

Fig. 1. The expressions of ER mRNAs in paired primary cultured eutopic and ectopic stromal cells were detected by RT-PCR. (A) Representative result of RT-PCR showing mRNA expression of ERα and ERβ in stromal cells from a woman with endometriosis. (B and C) After being normalized to 18S, the mRNA levels of ERα and ERβ (B) and ERα/ERβ ratio (C) are shown. Data are shown as mean and SEM from four patients. Paired *t* tests were used. $*p < 0.05$ compared to the ectopic group. $**p < 0.01$ compared to the ectopic group. Ec = ectopic; ER = estrogen receptor; Eu = eutopic; mRNA = messenger RNA; RT-PCR = reverse transcription-polymerase chain reaction.

and ERβ protein level was also decreased in ectopic endometriotic stromal cells (Fig. 2C). The protein level of HIF-1α increased in ectopic endometriotic stromal cells as the results of our previous study [7].

Regulation of ER expression by HIF-1α

Next, we tested whether different expressions of ERs in stromal cells were regulated by HIF-1α under hypoxia. HIF-1α was knocked down by siRNA. Transfection of siRNA targeted at HIF-1α (Si HIF-1α) abolished ERβ mRNA expression in eutopic stromal cells under hypoxia condition, but reversed ERα mRNA expression in both control and hypoxia status. To show the change of ER expressions by hypoxia is a gene-specific event, a HIF-1α inducible gene, PDK-1, was used as a control. As expected, the level of PDK-1 mRNA was upregulated under hypoxia, and this expression was abolished when HIF-1α was knocked down. However, sequenced scrambled siRNA (Sr HIF-1α) was not effective at demonstrating these changes (Fig. 3). Taken together, the data demonstrated that hypoxia-induced HIF-1α may regulate the expression of ERα and ERβ.

Discussion

When endometrial debris retrogrades to peritoneal cavity, the first stress it faces is hypoxia. One response of stromal cells to hypoxia is through HIF-1-mediated gene expression. To investigate whether the promoter activities of ERα and ERβ might be regulated by HIF-1α, the promoter sequences of ERα and ERβ were retrieved from human genome database (Ensembl). The analysis of human ER promoters using a bioinformatic approach identified three candidate HREs on the promoter of ERβ and one on the promoter or 5′ untranslated region of ERα. Therefore, it is hypothesized that accumulated HIF-1α might bind to candidate HREs of human ER genes to regulate the transcription of ERs after retrograded endometrial debris exposed to hypoxia in peritoneal cavity.

The expression of ERα and ERβ in endometriotic tissue is still controversial. Using unpaired samples in most of the studies, that means comparing the expression of ERs in eutopic and ectopic endometriotic tissues from different individuals, may lead to the discordant results of ER expression in endometrium and ectopic endometriotic tissue. Since different genetic backgrounds introduce great variations to the

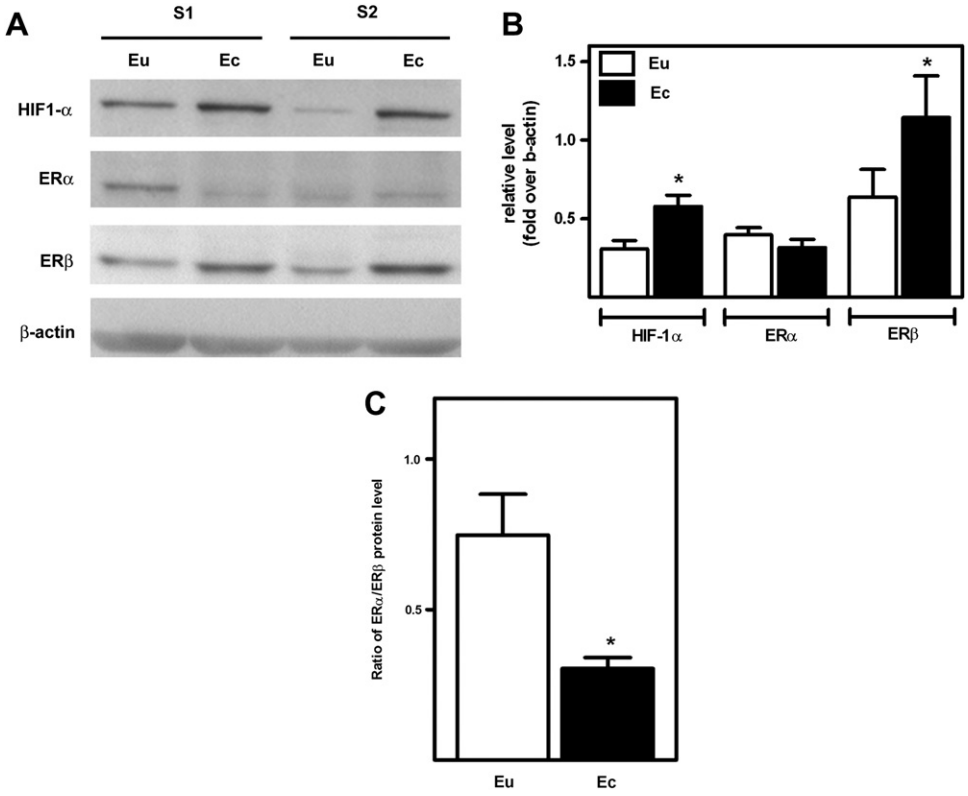

Fig. 2. The protein expressions of ER in paired primary cultured eutopic and ectopic stromal cells were detected by Western blot. (A) Representative Western blot showing protein expressions of HIF-1α, ERα and ERβ in stromal cells from women with endometriosis. S1 and S2 denote different patients with endometriosis. (B and C) After being normalized to β-actin, the protein levels of HIF-1α, ERα and ERβ (B) and ERα/ERβ ratio (C) are shown. Data are shown as mean and SEM from five patients. Paired *t* tests were used. $*p < 0.05$ compared to ectopic group. Ec = ectopic; ER = estrogen receptor; Eu = eutopic; HIF-1α = hypoxia inducible factor-1α.

levels of gene expression, it is necessary to precisely determine the difference of ERs in paired eutopic and ectopic endometriotic tissues. Our study used paired samples to reduce this inconsistency (Figs. 1 and 2). The mRNA expression of ERβ was significantly higher in ectopic endometriotic stromal cells than in eutopic stromal cells, and the ratio of ERα/ERβ was decreased in ectopic stromal cells (Fig. 1). Similar results were shown in protein levels. (Fig. 2).

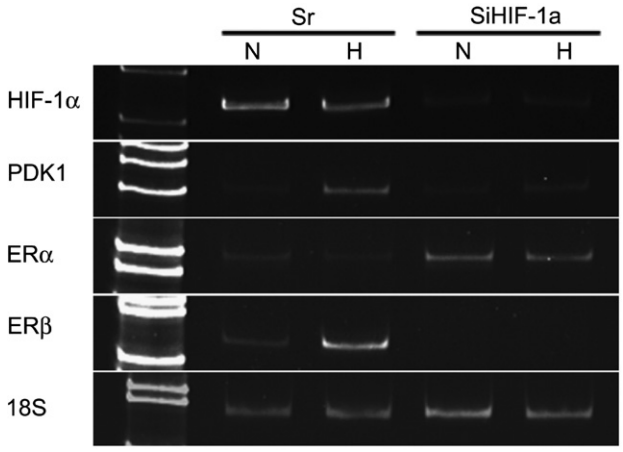

Fig. 3. A representative gel picture showing the result of transfection of siRNA targeted at HIF-1α (Si HIF-1α). The expression of HIF-1α, PDK-1, ERα, ERβ and 18S were detected by RT-PCR. ER = estrogen receptor; HIF-1α = hypoxia inducible factor-1α; PDK-1= pyruvate dehydrogenase kinase isozyme 1; RT-PCR = reverse transcription-polymerase chain reaction; siRNA = short interference RNA.

ERα transcriptional repression and its protein down-regulation by hypoxia are regulated by distinct mechanisms in breast cancer. Hypoxia induces proteasome-dependent down-regulation of ERα in MCF-7 human breast cancer cells [12]. In response to stabilization of the HIF-1α protein under hypoxia, ERα repression at the transcriptional level results from decreased recruitment of RNA polymerase II at the proximal promoter of the ERα gene in MCF-7 human breast cancer cell lines [13]. In our study, HIF-1α regulated expressions of ERα and ERβ in stromal cells may be mediated in a different way. Hypoxia increased ERβ mRNA expression and this effect was abolished by knocking down of HIF-1α (Fig. 3). Moreover, knockdown of HIF-1α increased ERα mRNA level no matter in normoxia or hypoxia (Fig. 3). It suggested HIF-1α may regulate ERα and ERβ transcription.

Hypoxia is an important stress for endometriosis. Eutopic endometrial and ectopic endometriotic stromal cells may have differential responses to hypoxia stress. The aberrant leptin expression in endometriotic stromal cells is induced by elevated HIF-1α [7]. Decreased expression of dual specificity phosphatase-2 (DUSP2) is found in endometriotic stromal cells [14]. Suppression of DUSP2 by HIF-1α in eutopic

stromal cells causes increased cyclooxygenase-2 gene sensitivity to interleukin-1β stimulation, which resembles endometriotic stromal cell characteristics shown in our previous study [15]. In this study, we confirmed that HIF-1α had a significant and direct effect of ERs expression in endometrial stromal cells. More studies are needed to clarify the mechanism of the involvement of hypoxia factors in mediating estrogen action via different ER expression in endometriosis. The results of this study will provide more understanding of how endometriosis is established and the mechanism of the switch from eutopic to ectopic stromal cells.

Acknowledgments

The authors thank Miss Mei-Feng Huang for technical assistance. This work was financially supported by grants from the Natural Science Council of Republic of China (NSC100-2314-B-006-012) and National Cheng-Kung University Hospital (NCKUH-99-04007) to M.H.W.

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