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New insights into the efficacy of SR-16234, a selective estrogen receptor modulator, on the growth of murine endometriosis-like lesions

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Problem: To evaluate the effects of SR-16234 (SR), a selective estrogen receptor modulator (SERM), on murine endometriosis-like lesions.

Method of study: BALB/c mice (n = 53) were used to establish the murine endometriosis model. Ovariectomized, estradiol replaced, 6-week-old murine endometriosis model were injected with lipopolysaccharide (LPS) with or without SR (1 mg/kg/d) or vehicle, over a period of 4 weeks. Upon treatment completion, the endometriosis-like lesions that developed in the abdominal cavity of mice were counted, measured, and collected. Gene expression of inflammatory cytokines and estrogen receptor (ER) in the lesions was assessed by real-time RT-PCR. Immunohistochemical analysis was used to evaluate the effect of SR on cell proliferation, angiogenic activity, inflammation, and NF-κB phosphorylation.

Results: Treatment with SR significantly reduced the total number and size of lesions per mouse without inducing endometrial growth. In addition, SR downregulated LPS-enhanced *Vegf*, *Il-6*, *Ptgs-2*, and *Ccl-2* and ER mRNA expression in endometriosis-like lesions. Immunohistochemical analysis demonstrated a decrease in percentage of positive cells of Ki67, and intensity and rate of positive cells of ERα, CD3, F4/80, PECAM by SR treatment. SR also decreased the expression of NF-κB p65 and phospho-NF-κB p65.

Conclusion: SR has a regressive effect on the development of murine endometriosis-like lesions.

KEYWORDS

estrogen receptor, lipopolysaccharide, murine endometriosis model, selective estrogen receptor modulators, SR-16234 (SR)

1 | INTRODUCTION

Endometriosis is defined as an ectopic growth of endometrial tissues outside the uterine cavity, and is associated with pain and infertility. The pathogenesis of endometriosis involves several mechanisms, including cell proliferation and differentiation, apoptosis, migration, adhesion and invasion, inflammation, and neuroangiogenesis.[1] It is considered as an estrogen-dependent disease in which two estrogen receptor (ER) isoforms, ERα and ERβ, play an important role. Until now, distinctive ER expression profile, a higher ERβ, and a lower ERα expression in human endometriotic tissues, which is in the inverse relationship in the endometrium, has been proposed as a major background of estrogen action in endometriosis.[2] In recent study, ERα and ERβ were expressed simultaneously and almost at a comparable

level in human endometriotic cells.[3] Endometriotic tissue contains not only ER but also aromatase, an enzyme that catalyzes the conversion of androgens to estrogens, suggesting that local estrogen production may increase the estrogen concentration.[4,5] Thus, the current medical therapies for endometriosis aim to decrease ovarian estrogen production and or counteract estrogen effects with the use of GnRH agonists, progestins, danazol, and oral contraceptives (OCs). However, due to their limited effectiveness and side-effects, alternative therapies that have fewer side-effects in estrogen target tissues are needed. Emerging newer treatment options include aromatase inhibitors, selective estrogen receptor modulators (SERMs), selective progesterone receptor modulators (SPRMs), and anti-angiogenic agents.[6]

SERMs, used for treatment of infertility, breast cancer, and osteoporosis prevention, have a spectrum of ER agonistic/antagonistic activity depending on the target tissue. They interact with ER and block the hormonal signaling pathway,[7] and have been considered for using in the treatment of endometriosis due to their antiproliferative effects on the endometrium.[8] Tamoxifen (a first generation SERM) reduced the incidence of breast cancer in high-risk patients, but has the increased risk of development and progression of endometrial cancer.[9] Unlike tamoxifen, raloxifene (a second-generation SERM) do not increase the risk of endometrial cancer and is approved for long-term treatment in the prevention of osteoporotic fractures and for the reduction in invasive breast cancer risk in postmenopausal women.[10,11] One randomized study evaluated whether raloxifene was effective in the treatment of chronic pelvic pain in women with endometriosis. However, this trial had to be terminated early when the raloxifene group experienced pain and had second surgery significantly sooner than the placebo group.[12]

Newer generation SERM, bazedoxifene (BZA), also prevented and treated post-menopausal osteoporosis without adverse stimulation of the breast and endometrium.[13] Recently, a tissue-selective estrogen complex (TSEC), which combines BZA and conjugated estrogen (CE), was developed to maximize the tissue-specific effects of SERM and minimize their side-effects. TSEC has an anti-estrogenic effect on the growth of breast cancer cells and prevents the development of endometrial hyperplasia in post-menopausal women.[14-16] The suppressive effect of TSEC on ectopic lesion growth in mice with surgically induced endometriosis has also been reported.[17,18] However, the effectiveness of BZA alone or TSEC on endometriosis in humans has yet to be evaluated. Other several SERMs have also been studied in the experimental animal model of endometriosis, including raloxifene,[17,18] LY-2066948,[19] TZE-5323,[20] BZA[21] to have reported efficacies on the regression of endometriosis lesions. In this study, we establish the homologous murine endometriosis model by transplanting uterine tissue,[22] and evaluate the effects of SR-16234 (SR) on murine endometriosis-like lesions.

SR is a SERM, which is reported to have ERα antagonistic activity with a weak partial agonist activity to ERβ receptor. Comparing with other SERMs, which have ERα partial agonistic activity, SR is predicted to yield a superior effect in humans due to its pure antagonistic action on ERα.[23,24] SR may be an ideal candidate for treating endometriosis because of its selective ER agonistic/antagonistic activity depending on the target tissue. Several studies have also evaluated the utility of SR, which is also known as TAS-108, in patients with metastatic breast cancer,[24-26] and its safety and tolerability in normal healthy post-menopausal patients.[27] Recently, an open-label clinical trial reported the efficacy and safety of SR in women with symptomatic endometriosis[28] and suggested that SR may alleviate endometriosis-associated dysmenorrhea and pelvic pain at 40 mg daily dosage by oral administration. In this study, we investigated the effects of SR on the development of murine endometriosis-like lesions.

2 | MATERIALS AND METHODS

2.1 | Animals

All procedures were performed in accordance with protocols approved by the Animal Care and Use Committee of Tottori University Faculty of Medicine. Four-week-old BALB/c female juvenile mice were purchased from Japan SLC (Shizuoka, Japan) and allowed to have at least 1 week of acclimation to the laboratory's conditions before initiating the experiment.

2.2 | Induction of endometriosis

All mice were ovariectomized and then injected subcutaneous (s.c) with estradiol (E2) (0.5 μg/mouse; Fuji Pharma, Tokyo, Japan) once per week for 6 weeks. Two weeks after ovariectomy, the whole uteri from donor mice (n = 18) were removed *en bloc* after euthanasia, washed in sterile saline, divided into two horns, slit with a linear incision longitudinally, and minced (approximately 0.5 mm in diameter) with dissecting scissors. To create experimental endometriosis, recipient mice (n = 35) were anesthetized using pentobarbital sodium (50 mg/kg). Laparotomy was performed by 0.5 cm subabdominal midline incision, and minced donor tissue (1:2 donor uterus to host ratio) in 250 μL saline was injected into the peritoneal cavity of recipient mice. Finally, the peritoneum was sutured.

2.3 | Treatment

After induction, recipient mice were treated with vehicle (n = 10; saline, intraperitoneal (i.p), twice a week) or lipopolysaccharide (LPS) without SR (n = 10; 0.05 mg/kg, i.p; twice a week) or LPS (0.05 mg/kg, i.p; twice a week) with SR (n = 10; 1 mg/kg; s.c, daily) in corn oil or LPS with BZA (Bazedoxifene acetate, Cayman Chemical, USA) (n = 5; 1 mg/kg; s.c, daily) in corn oil for 4 weeks. At the day of donor's inoculation of uterine tissues, first LPS injection was done, and 1 day later, SR and BZA were started given. In this study, we also used the LPS to promote the pelvic inflammatory process[29] and investigated the additive effect between E2 and LPS on pelvic inflammation and growth of murine endometriosis-like lesions. We tested the various amount of LPS, set the lowest effective dose of LPS (0.05 mg/kg)

and injected twice a week after considering the short half-life of LPS (data not shown). The dosages of SR and BZA were determined according to the previous study using ovariectomized rat model (unpublished data), and the literature evaluated the efficacy of BZA, respectively.[30] SR was provided by Nobel pharma Co. (Tokyo, Japan).

2.4 | Tissues collection

After 4 weeks of treatment, mice (11 weeks of age) were killed and the peritoneal cavities of the mice were thoroughly inspected. Uteri and endometriosis-like lesions were carefully removed, weighed, measured, and photographed to document in situ images using a microscope. The surface area (square millimeters) of each endometriosis-like lesion was calculated by multiplying length (millimeters) by width (millimeters). Using Image-J (NIH, Bethesda, MD, USA), the surface area of each lesion could be calculated by the manual measurement of perimeter. Then, the lesions were placed in formalin or in RNAlater™ solution (Life Technologies, Tokyo, Japan), and frozen immediately at −80°C for later use.

2.5 | Real-time RT-PCR

RNA purification, cDNA synthesis, and qPCR were performed as described previously. [22] Total RNA was extracted from the endometriosis-like lesions and the corresponding eutopic endometrial tissues using RNeasy Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's instructions. RNA (1 μg) from the tissues was reverse transcribed into complementary DNA. The mRNA levels were quantified using the ABI 7900 HT real-time PCR system (Applied Biosystems, Tokyo, Japan). The ABI TaqMan probes for each gene and TaqMan mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control Reagents (Applied Biosystems) were used. Then, gene expression of the major inflammatory cytokines, murine vascular endothelial growth factor (*Vegf*), Interleukin (*Il*) -6, monocyte chemotactic protein-1 (*Ccl-2*: Mcp-1), prostaglandin-endoperoxide synthase 2 (*Ptgs-2*: Cox-2), ERα, and ERβ in the endometriosis-like lesions, and eutopic endometrium were evaluated. Expression was normalized to the expression of GAPDH from the same sample. All samples were tested in triplicate, and each run included no-template and no-RT controls.

2.6 | Immunohistochemistry (IHC)

IHC was performed on formalin-fixed, paraffin-embedded endometriosis-like tissue which is cut into 5-μm sections. Slides were deparaffinized through a series of xylene and ethanol washes. Immunohistochemical analysis was performed as previously described.[22] The primary antibodies used were ERα (Abcam, Tokyo, Japan), ER β (Abcam), Ki67 (Abcam), Toll-like receptor (TLR) 4 (the receptor for LPS; Novus Biologicals, Littleton, CO, USA), CD3 (T cell marker, Abcam), F4/80 (macrophage cell marker, Abcam), PECAM (endothelial cell marker, Abcam), NF-κB p65 (Abcam), and phospho-NF-κB p65 (phosphor S536, Abcam). All slides were processed simultaneously for each primary antibody. Proliferative activity was evaluated by counting Ki67-positive and Ki67-negative nuclei of all epithelial and stromal cells. The ratios of Ki67-positive cells were averaged over 3 fields in a single section of each lesion. The percent of cells was determined with each staining intensity category (graded as 0; no staining, 1; weak, 2; medium, 3; strong), and H-score was determined using the formula 1 × (% of 1 + cells) + 2 × (% of 2 + cells) + 3 × (% of 3 + cells). The ranges of score were from 0 to 300. Negative control slides were incubated similarly, but the primary antibody was replaced with PBS.

2.7 | Statistical analysis

All experiments were repeated a minimum of three times. Statistical significance was assessed using JMP software (SAS Institute, Cary, NC, USA). Data were analyzed using one-way ANOVA, followed by Fisher's protected least significant differences post hoc test, and presented as means with SEMs. The mean ΔCt was calculated from individual ΔCt values obtained from a minimum of three replicates. ΔΔCt was calculated as the difference between the mean ΔCt values of the experimental and control samples. The fold change of gene expression in each sample relative to a control was computed as $2^{-\Delta\Delta Ct}$. $P < 0.05$ was considered to be statistically significant.

3 | RESULTS

3.1 | SR-16234 reduces the growth of murine endometriosis-like lesions

Most of the endometriosis-like lesions were developed around the peritoneal incision and the intestinal membrane. Collected specimens are shown in Figure 1A. LPS administration for 4 weeks significantly increased the extent of endometriosis-like lesions compared with the vehicle group. Treatment with SR led to a reduction in the total number (LPS: 11 ± 1.2 vs SR: 5.6 ± 0.8/mouse: $P < 0.05$), and weight (LPS: 193.6 ± 22.6 vs SR: 97.1 ± 26.9 mg/mouse: $P < 0.05$) of all the endometriosis-like lesions per mouse, whereas the BZA attenuated only the number of lesions (Figure 1B,C). The surface area of endometriosis-like lesions was significantly lower in SR-treated mice, 59.7 ± 15.9, and 112.9 ± 11.3 mm^2 in the SR treatment, and LPS alone group, respectively ($P < 0.05$; Figure 1D). On the other hand, no significant differences in uterine weight (Figure 1E), and body weight (data not shown) were found between the groups.

3.2 | Expression of inflammatory-associated cytokines and estrogen receptor after SR-16234 treatment

Real-time RT-PCR analysis demonstrated SR and BZA treatment reduced the LPS-enhanced *Vegf*, *Il-6*, *Ptgs-2* and *Ccl-2* mRNA expression in endometriosis-like lesions ($P < 0.05$; Figure 2A), whereas the expression in eutopic endometrium was not different (Figure 2B). ERα and ERβ mRNA expression in endometriosis-like lesions, and

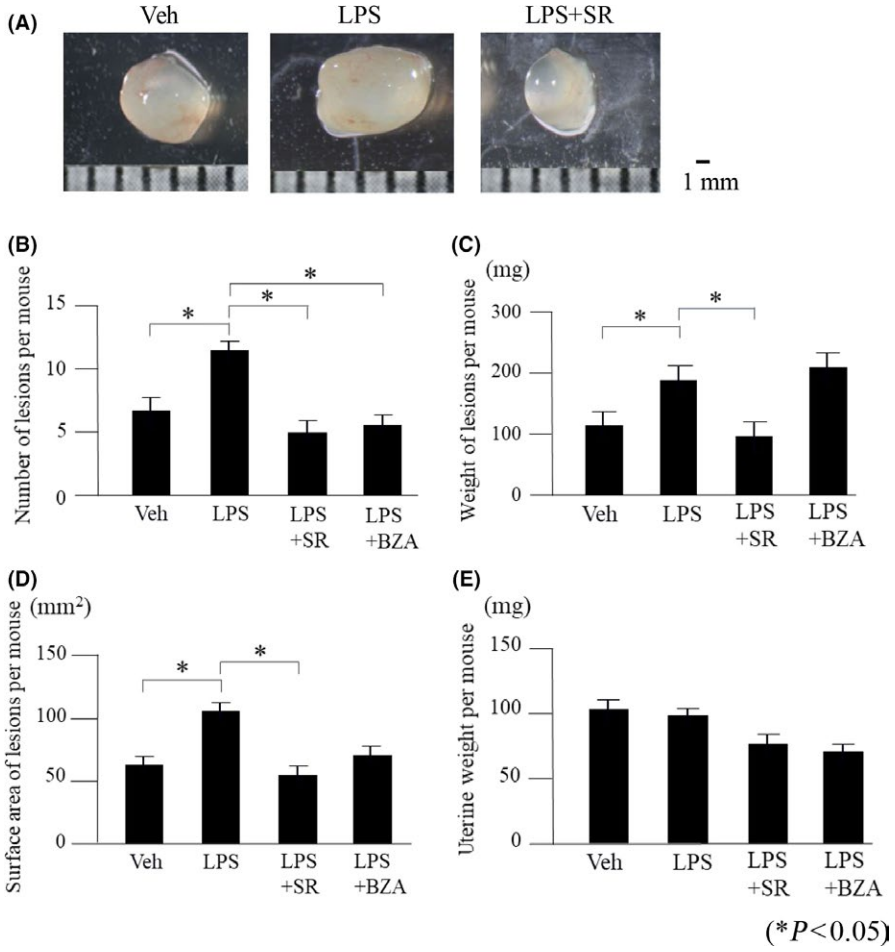

FIGURE 1 Effects of SR-16234 (SR) on the growth of murine endometriosis-like lesions, 4 wk after treatment. A, Representative ectopic lesions isolated from each group of mice, (B) total number, (C) weight, (D) surface area of lesions, and (E) uterine weight are shown. Veh, vehicle, BZA, bazedoxifene. Bars represent the average ± SEM. (*P < 0.05)

eutopic endometrium were also analyzed. In lesions, levels of ERα and ERβ were increased by the addition of LPS, and SR significantly decreased those mRNA expressions. BZA treatment reduced only ERα mRNA (P < 0.05; Figure 3A). In eutopic endometrium, the effect of LPS on ERα and ERβ was insignificant, and there were no significant differences by SR or BZA treatment (data not shown). To confirm the changes in ERα mRNA expression, immunohistochemical analysis was carried out. By H-score analysis, SR decreased the LPS-enhanced expression of ERα (epithelia: LPS: 12.2 ± 1.97 vs SR: 4.0 ± 1.3, stroma: LPS: 10.0 ± 2.0 vs 1.5 ± 0) in endometriosis-like lesions (P < 0.05; Figure 3B). In contrast, lesions formed in the vehicle and SR-treated mice expressed low levels of ERβ, and ERβ expression was not significantly different by treatment (data not shown).

3.3 | Effect of SR-16234 on cell proliferation, angiogenesis, inflammation, and NF-κB phosphorylation

To evaluate the proliferative activity of lesions, the ratio of Ki67-stained cells in epithelial and stromal cells was calculated. The percentage of Ki67-positive cells increased after LPS treatment, and SR diminished the LPS-induced proliferative effects (epithelia: LPS: 17.7 ± 3.1 vs SR: 5.3 ± 1.1%, stroma: LPS: 16.5 ± 2.9 vs SR: 4.6 ± 2.5%; Figure 4A). SR also repressed the expression of TLR4, a receptor recognizing LPS, in both epithelial and stromal cells (epithelia: LPS: 19.1 ± 2.4 vs SR: 8.3 ± 0.6, stroma: LPS: 13.3 ± 2 .4 vs 2.9 ± 0.3) (P < 0.05; Figure 4B). The intensity of CD3, F4/80, and PECAM in endometriosis-like lesions after SR treatment were also analyzed. SR significantly attenuated the LPS-enhanced intensity and rate of positive cells of CD3 in T cells (LPS: 5.2 ± 1.1 vs SR: 1.6 ± 0.2: P < 0.05), F4/80 in macrophages (LPS: 21.6 ± 2.7 vs SR: 6.6 ± 0.9), and PECAM (LPS: 19 ± 4.5 vs SR: 6 ± 0.7: P < 0.05) in endothelial cells (Figure 4C). SR treatment also attenuated the LPS-enhanced NF-κB p65 and phospho-NF-κB p65 expression detected in epithelial and stromal cells of endometriosis-like lesions (Figure 4D).

4 | DISCUSSION

SR-16234 is a novel alternative treatment for endometriosis. In this study, we demonstrated that SR effectively suppressed the growth, and inflammatory-associated genes expression in endometriosis-like lesions without inducing endometrial growth. We have compared the efficacy of SR with BZA, which is third generation SERM. Interestingly, SR seemed to have a more repressive effect than BZA in the growth of murine endometriosis-like lesions. We have also shown the antiproliferative, anti-inflammatory, and anti-angiogenic

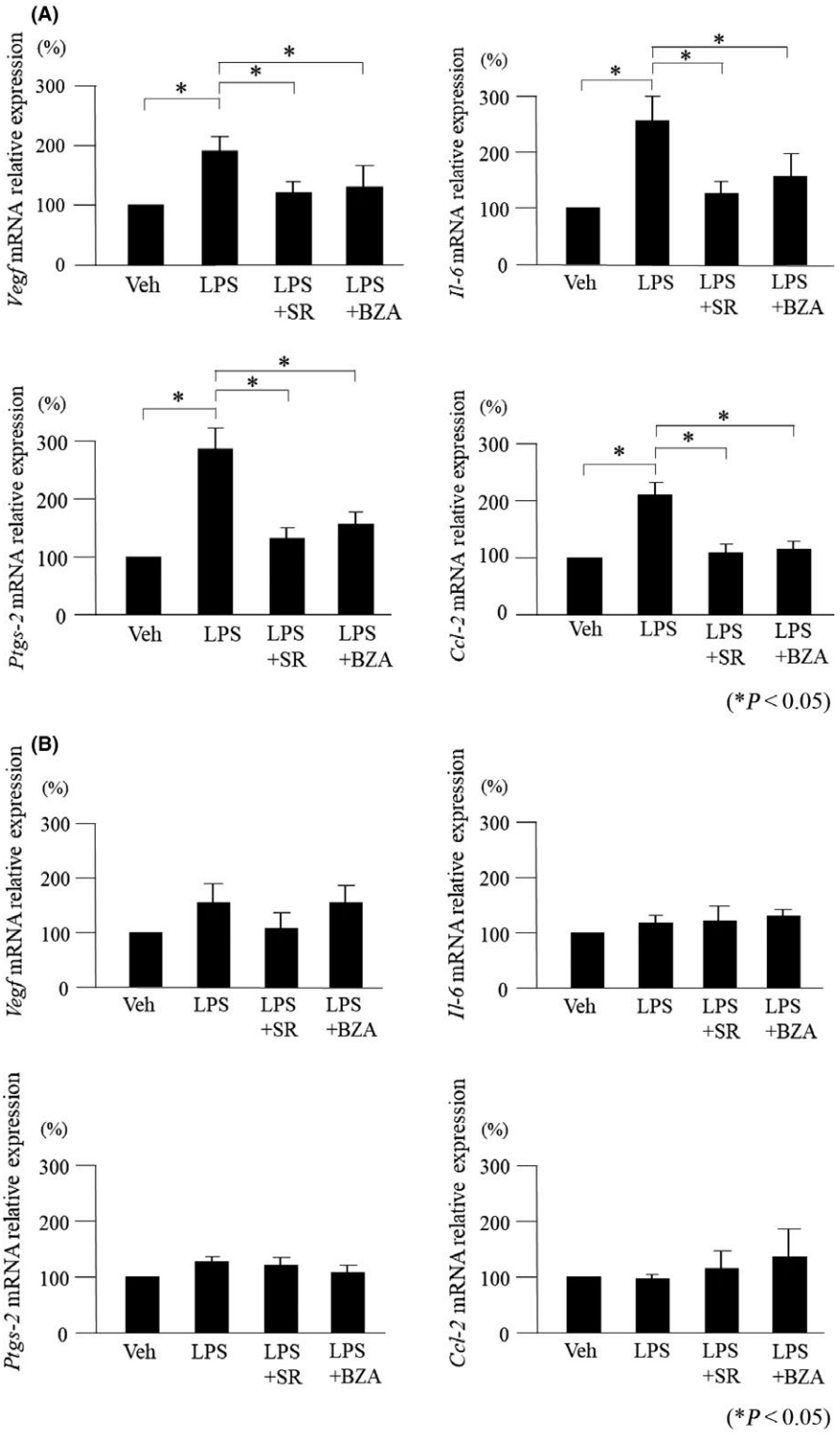

FIGURE 2 Real-time RT-PCR demonstrates expression of inflammation-associated genes (*Vegf*, *Il-6*, *Ptgs-2*, and *Ccl-2)* in (A) murine endometriosis-like lesions, and (B) eutopic endometrium. Veh, vehicle, SR, SR-16234, BZA, bazedoxifene. Bars represent the average ± SEM. (*P < 0.05)

activity of SR. SR also inhibited LPS-enhanced expression of NF-κB activation. Several in vitro and in vivo studies have shown that NF-κB inhibition reduced the development of endometriosis by diminishing inflammation and cell proliferation, and increasing apoptosis of endometriotic cells.[31-33]

Beyond the estrogen-dependent nature of endometriosis, the distinct ER expression profile in endometriosis has also been demonstrated.[2,3,34] In humans, so far, a higher ERβ, and a lower ERα expression in endometriotic tissues, which is in the inverse relationship in the endometrium, has been reported.[2]

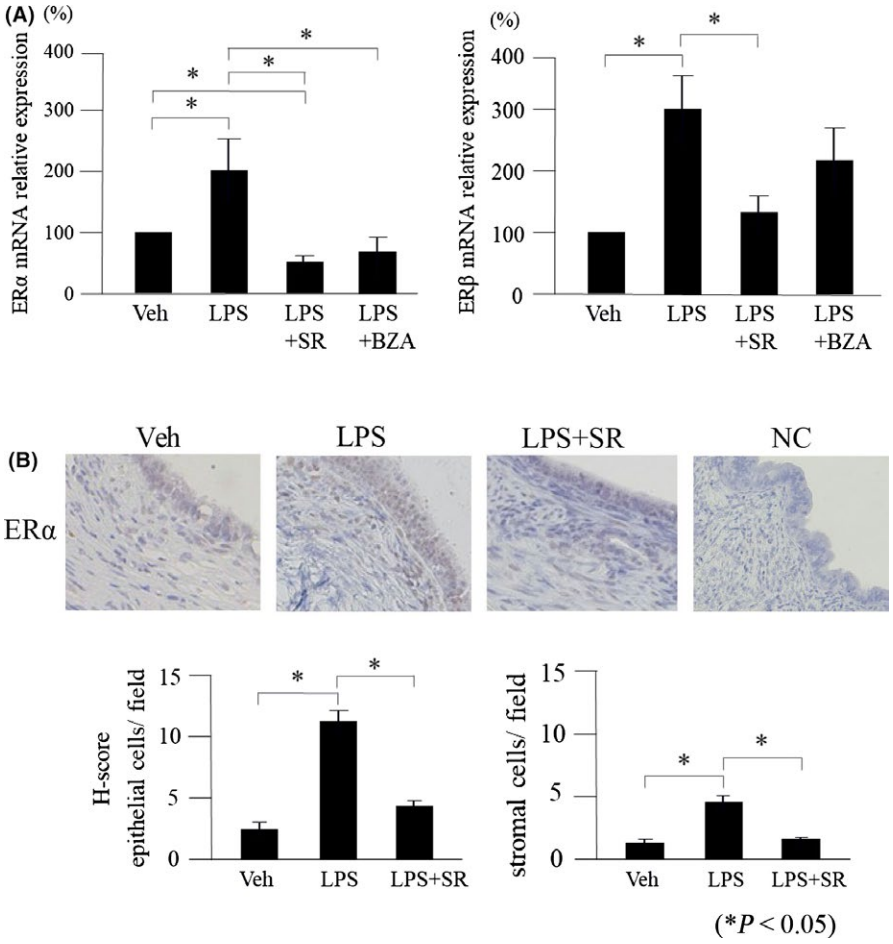

FIGURE 3 Expression of estrogen receptor (ER) after SR-16234 (SR) treatment. A, real-time RT-PCR analysis of ERα and ERβ in murine endometriosis-like lesions. B, Immunohistochemical analysis demonstrates expression of ERα in endometriosis-like lesions. Representative immunostained slides and semi-quantitative analysis (H-score) are shown. Veh, vehicle, BZA, bazedoxifene NC: negative control. Original magnification ×400. Bars represent the average ± SEM. (*$P < 0.05$)

In recent study, ERα and ERβ were expressed simultaneously, and almost at a comparable level in human endometriotic cells.[3] Studies of mice lacking two ER subtypes have also provided important insights into the function of ERα and ERβ in the development of murine endometriosis-like lesions[35,36] Burns and colleagues demonstrated that ERα is required for attachment, inflammation, and proliferation of ectopic lesions.[35] The gain of ERβ function study also revealed that ERβ enhances adhesion, invasion, and inflammation signaling for the establishment of murine ectopic lesions.[2] However, the available evidence regarding the specific contribution of each ER isoform in murine endometriosis-like lesions is contradictory.[34] In the present study, we showed that the mRNA expression of the ERα and ERβ in murine endometriosis-like lesions were significantly attenuated by SR treatment. By H-score analysis, SR significantly decreased the expression of ERα in epithelial and stromal cells of murine lesions. Intriguingly, in our study, by both real-time RT-PCR, and IHC, lesions formed in all groups expressed low levels of ERβ. A possible explanation is that the endometriosis-like lesions we examined were originated from mouse uterine tissue, where ERα is dominant. We believe that it is difficult to detect the ERβ by IHC in mouse uterus and human uterus. It has also been demonstrated that ERβ protein is expressed in a limited number of normal and cancer tissue types, with the highest expression detected in ovary.[37] Other possibilities of differential regulation of ERβ in murine lesions between our study and previous reports[2,38] might be attributable to the use of different antibodies and pharmacological inhibition.

Currently, most of the medical treatment of endometriosis-associated pain is based on suppressing estrogen production, which creates a relatively hypoestrogenic environment that inhibits ectopic endometrial growth and prevents disease progression.[6] However, due to their estrogen-deficiency side-effects, alternative effective therapies that are appropriate for long-term use is needed, and certain ER modulators have been proposed to alleviate the clinical symptoms of endometriosis. SERMs display ER agonist and antagonistic effects in a tissue-specific profile. They interact with ER and block the hormonal signaling pathway, leading to a reduction in estrogen activity.[7] They might be used as an alternative endometriosis treatment due to its beneficial estrogen-like activities and reduced side-effects based on the different distributions and relative levels of ER subtypes in different estrogen target tissues.[8]

SR is a SERM which has estrogenic effects on bone and cardiovascular system and anti-estrogenic effects on breast and uterine tissues. In addition to its pure ERα antagonistic activity, SR act as

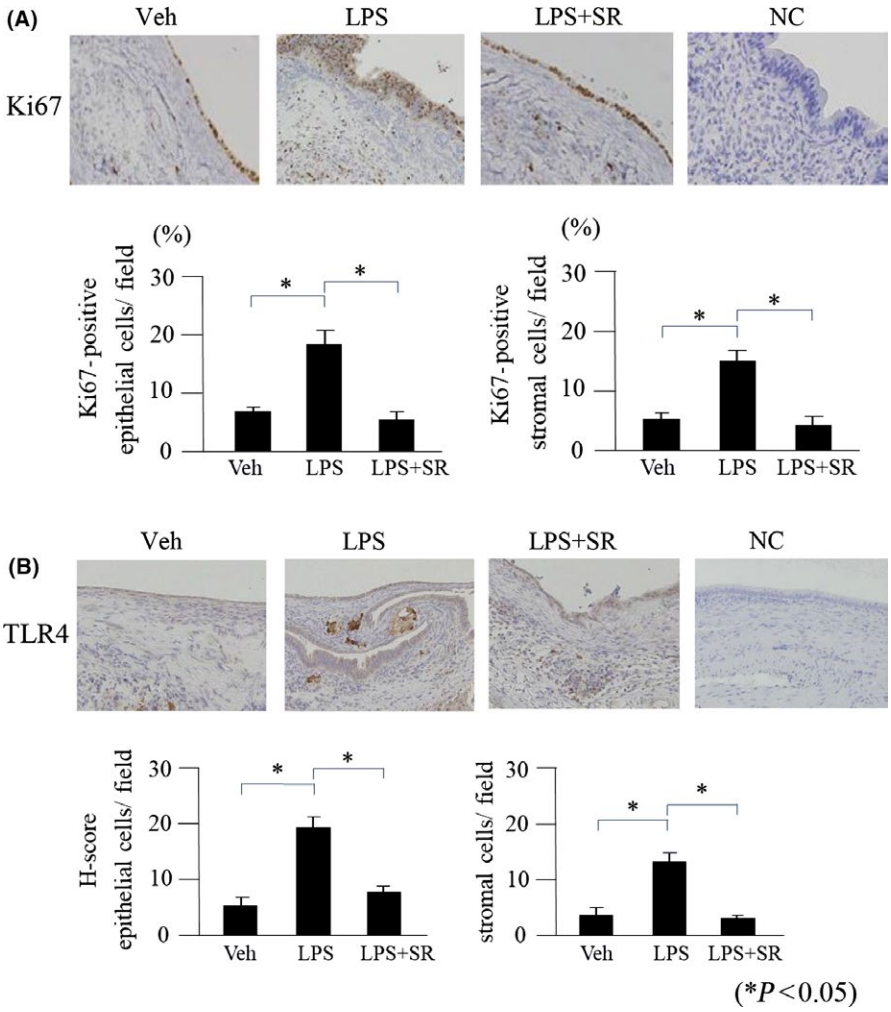

FIGURE 4 Effects of SR-16234 (SR) on the murine endometriosis-like lesions were assessed by immunohistochemical analysis. Representative immunostained slides and semi-quantitative analysis (H-score) are shown. A, Ki67 staining and the rate of positive cells in epithelium and stroma of lesions are shown. Results are expressed as percentage ± SEM. B, Expressions of TLR4; (C) CD3, F4/80, PECAM; and (D) NFκB-p65, phospho-NFκB-p65 were evaluated. Veh, vehicle, NC: negative control. Original magnification ×400. Bars represent the average ± SEM. (*P < 0.05)

ERβ partial antagonist in estrogen rich conditions like endometriotic tissue. In a previous study using ovariectomized rat models with high E2 supplementation, SR exhibited anti-estrogenic activity from the dose of 1 mg/kg/d in a dose-dependent manner.[24] Thus, in this study, instead of using natural estrous cycles, we used the ovariectomized mice with E2 supplementation, and investigated the effects of SR at a dosage of 1 mg/kg/d on growth of endometriosis-like lesions and uteri. Comparing with other SERMs, which have ERα partial agonistic activity, SR is predicted to yield a superior effect in humans due to its pure antagonistic action on ERα. It may be more effective for endometriosis-related pain. In addition, SR has high binding affinity to ERα and ERβ, and its action on ERβ is also characteristic among SERMs. Its distinctive ERβ partial agonistic activity may also provide advantages for some organs such as bone or cardiovascular systems.[23,24] Recent clinical trial have also shown that oral administration of SR 40 mg once daily for 12 weeks significantly decreased the pelvic pain and dysmenorrhea scores, stiffness of Douglas' pouch, and limitation of uterine movement in endometriosis patients. No serious adverse events were reported in that trial. SR seems to be the first reported SERM with such excellent clinical efficacies in endometriosis-associated symptoms.[28] It might be used as an alternative therapy to reduce the symptoms of estrogen deprivation, in contrast to current therapeutics that suppress systemic estrogen levels.

Local inflammatory reaction in the peritoneal environment is also considered one of the contributing factors in the pathogenesis of endometriosis.[39] Studies have been demonstrated the effects of inflammatory mediators, such as LPS or combined effects of E2 and LPS on promoting proinflammatory response in pelvis and growth of endometriosis.[29,40,41] Zhao et al revealed the two novel ER ligands are effective in suppressing murine and human endometriotic cell growth by the dual suppression of estrogenic and inflammatory activity. Thus, considering the synergistic effect between E2 and LPS, we also used the LPS to promote the pelvic inflammatory process. Consistent with previous study,[22] our data showed that LPS increased the growth and expression of inflammatory-associated genes in murine endometriosis-like lesions, but not in the eutopic endometrial tissues. We also showed that SR reduced the expression of TLR4, which is an essential receptor for LPS recognition. In addition to typical estrogen suppressing agent, combined targeting of E2 and LPS could be useful in the treatment of endometriosis. Moreover, as shown in Figure 3, LPS enhanced the expression of ERα and ERβ in the endometriosis-like lesions. Although the information whether the inflammation

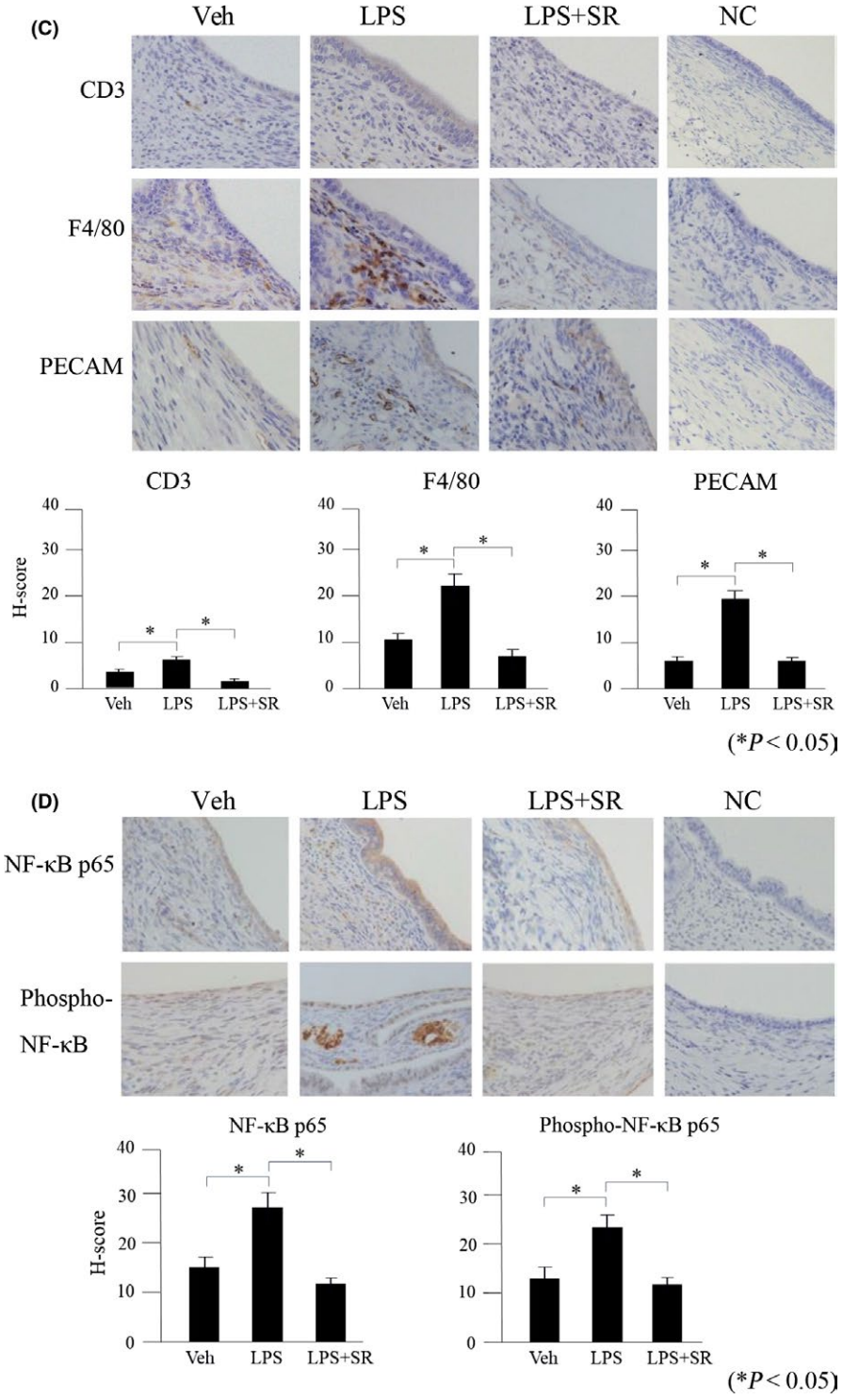

FIGURE 4 Continued

affects the differential expression of ER isoforms is limited, it has been demonstrated that inflammatory status increases the aromatase and steroid receptor expression in endometriosis. [42] However, in this study, because of the excessive estrogen environment, we could not study the signaling pathways leading to anti-inflammatory response induced by SR in endometriosis-like lesions.

Collectively, our findings reveal that SR is effective in suppressing murine endometriosis-like lesions growth by affecting key aspects of cell proliferation, angiogenesis, and inflammatory activity, which are crucial for establishment, progression, and recurrence of the disease. Further experiments using animal models as well as clinical trials will be helpful to explore the effectiveness of SR for treating endometriosis.

CONFLICT OF INTEREST

The authors disclose no conflict of interests.

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