Physiologic activation of nuclear factor kappa-B in the endometrium during the menstrual cycle is altered in endometriosis patients

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Objective: To evaluate nuclear factor kappaB (NF-κB) activation and NF-κB–p65 subunit activation, immunolocalization, and expression in the endometrium of healthy women and endometriosis patients throughout the menstrual cycle.

Design: Prospective observational study.

Setting: Affiliated hospital and university research laboratory.

Patient(s): Twenty-four healthy women and 24 endometriosis patients.

Intervention(s): Menstrual, proliferative, and secretory endometrial biopsies.

Main Outcome Measure(s): Assessment of NF-κB and p65 activation by protein-DNA binding assays and p65 localization and expression by immunohistochemistry.

Result(s): Total NF-κB–DNA binding was constitutive and variable in human endometrium accross the menstrual cycle. Healthy women (physiologic conditions) showed higher p65-DNA binding in proliferative than in menstrual and secretory endometrium. Conversely, in endometriosis patients, p65–DNA binding was higher in proliferative and secretory endometrium than in menstrual endometrium. Endometrial epithelial cells showed higher p65 expression level score than endometrial stromal cells.

Conclusion(s): NF-κB activity is constitutive, physiologic, and variable in human endometrium. The physiologic cyclic p65 activation pattern was altered in endometriosis patients, showing no cyclic variation between the proliferative and secretory phase of the menstrual cycle. The absence of decreased p65 activity in secretory endometrium from endometriosis patients is concurrent with progesterone resistance and could participate in endometrial biologic alterations during the implantation window in endometriosis patients. (Fertil Steril® 2012;97:645–51. ©2012 by American Society for Reproductive Medicine.)

Key Words: Endometrium, endometriosis, NF-kappaB, p65, menstrual cycle

Nuclear factor kappaB (NF-κB) is a family of transcription factors modulating hundreds of genes involved in inflammation, cell proliferation, apoptosis, invasion, angiogenesis, and other cell processes (1–4). The classic NF-κB pathway is activated by proinflammatory stimuli such as interleukin-1β, tumor necrosis factor α, and lipopolysaccharide derived from

bacteria (2, 5, 6). Subsequently, phosphorylation of the NF-κB–coupled inhibitory protein (IκB) by IκB kinase β and its ubiquitination triggers IκB degradation by the proteasome, releasing p50/p65 dimers, which translocate to the nucleus and bind to DNA, activating the transcription of its target genes. Activation of p50/p65 dimers of NF-κB stimulates inflam-

mation, innate immunity and cell survival. Atypical pathways may also activate p50/p65 NF-κB dimers, and the nonclassic or alternative pathway of NF-κB activates p52/RelB NF-κB dimers, regulating lymphoid organogenesis, B-cell maturation, and humoral immunity (2, 5–10).

Multiple publications have strongly suggested participation of the NF-κB pathway in endometriosis pathophysiology (11–16). In vitro studies have shown positive regulation of growth factors and proinflammatory and antiapoptotic proteins mediated by NF-κB activation in human endometrial and endometriotic cells (17–32). In vivo research in animal models treated with NF-κB inhibitors has revealed reduction of endometriosis development by diminishing inflammation and cell proliferation and inducing apoptosis of

endometriotic cells (13, 33). Constitutive activation of NF-κB was shown to be increased in red endometriotic lesions relative to black endometriotic lesions in women (12), and iron-mediated NF-κB activation in pelvic macrophages and endometriotic cells has been proposed as a possible mechanism contributing to endometriosis establishment and maintenance (15, 16, 34). Basal NF-κB activity has been demonstrated in human endometrium and endometrial cells, and alterations of the NF-κB pathway activity were shown in eutopic endometrium of endometriosis patients (17, 18, 35–37). Endometrial NF-κB activation in the three different phases of the menstrual cycle in physiologic conditions and in endometriosis patients has not yet been evaluated.

The aim of the present study was to evaluate endometrial NF-κB activation in healthy women and in endometriosis patients, including the three phases of the menstrual cycle to gain further insight into cyclic regulation of NF-κB activity in physiologic conditions and disease. To assess classic and/or atypical NF-κB activation pathways, p65 peptide–DNA binding, intracellular localization, and expression were determined.

MATERIALS AND METHODS
Endometrial Biopsies

The use of human tissue for this study was approved by the Ethical Review Boards of the University of Chile, Hospital San Borja Arriarán, and Fondo Nacional de Desarrollo Científico y Tecnológico. Endometrial biopsies were obtained with the use of a Pipelle de Cornier biopsy curette (Laboratoire CCD) from 24 healthy women (ages 31–45 years, mean 37.4 ± 3.8) and 24 endometriosis patients (ages 22–46 years, mean 33.0 ± 6.5) not receiving hormonal treatment but undergoing surgery for tubal sterilization or endometriosis. Ten patients had endometriosis grade I–II, and 14 patients had endometriosis grade III–IV, according to the American Society for Reproductive Medicine classification (38).

Menstrual endometrial samples were obtained on day 1–3 of the menstrual cycle from eight healthy women (control subjects) and eight endometriosis patients. Proliferative endometrium was taken from eight control subjects and eight endometriosis patients on day 5–14 of the menstrual cycle. Secretory endometrium was obtained from eight control subjects and eight endometriosis patients on day 19–30 of the menstrual cycle. Endometrial phase of the biopsies were histologically confirmed according to standard criteria (39). Samples were immediately placed and transported on ice. A portion of each sample was fixed in 4% buffered formaldehyde and embedded in paraffin for dating and immunohistochemical staining. Another endometrial portion was stored at −80°C until use for protein extraction techniques.

Nuclear Protein Extraction

Endometrial biopsies were lysed in a Dounce homogenizer (Wheaton). Nuclear proteins from the endometrial tissue homogenate were extracted using NE-PER extraction reagents (Pierce Perbio Science) according to the manufacturer's protocol. Protease inhibitor cocktail (Sigma) was added to the nuclear extraction reagents (20 μL/mL). Phosphatase inhibitors were also added: 0.5 mmol/L sodium orthovanadate and 1 mmol/L sodium fluoride. Nuclear extracts were stored at −80°C until use. Protein concentrations were determined by using the Bradford method with a protein assay kit (Bio-Rad Laboratories). Sample freezing did not alter protein concentrations, as established previously (12).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) shows total NF-κB–DNA binding, including p50 and p65 homo- and heterodimers of NF-κB. NF-κB–DNA binding was tested with the consensus oligonucleotide of NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′, 3′-TCA ACT CCC CTG AAA GGG TCC G-5′) (Promega Corp.). The oligonucleotide was biotin labeled with Biotin 3′ End DNA Labeling Kit (Thermo Scientific) following the manufacturer's protocol. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology) according to the manufacturer's protocol. In brief, 5 μg nuclear proteins and 20 fmol biotin-labeled oligonucleotide were incubated for 20 minutes at room temperature (RT) in binding buffer (50 ng/μL poly(dI-dC), 50% glycerol, 1% NP-40, 1 mol/L KCl, 100 mmol/L $MgCl_2$, and 200 mmol/L EDTA) in a final volume of 20 μL. DNA-protein complexes were then loaded, adding 5 μL 5× loading buffer to each 20-μL binding reaction, onto a 5% nondenaturing polyacrylamide gel. The gel was run at a constant voltage of 100 V for 1 hour in 0.5× tris-borate-EDTA (TBE) buffer and binding reactions were then transferred to a nylon membrane (Pierce Biotechnology) for 1 hour at 100 V in 0.5× TBE. Crosslink of transfered DNA was performed for 15 minutes on a transilluminator at 312 nm. Biotin-labeled NF-κB nucleoprotein-oligonucleotide complexes were detected by chemiluminescence incubating the membranes for 15 minutes in blocking solution and 15 minutes in conjugate/blocking solution. Developing was done in the Discovery (Ultralum) imaging system. Adding an excess of unlabeled oligonucleotide to the reaction 30 minutes before adding the biotin-labeled probe checked the specificity of the binding reactions.

NF-κB (p65)–DNA Binding Immunodetection Assay

Activation of dimers containing the p65 subunit of NF-κB was determined using the TransAM kit (Active Motif) according to the manufacturer's instructions.

Briefly, DNA binding of p65 was investigated using a well plate with a coated oligonucleotide containing an NF-κB consensus-binding site (5′-GGGACTTTCC-3′). Five micrograms of nuclear proteins were incubated in the wells for 1 hour at RT. p65 monoclonal antibody (1:1,000) was added to each well and then horseradish peroxidase–conjugated secondary antibody (1:1,000) was added. After 100 μL developing solution was added to each well, the absorbance was examined on a spectrophotometer (Ultrospec 2100 Pro; Amersham Biosciences) at 450 nm with a reference wavelength of 655 nm. TPA+CI-stimulated Jurkat nuclear extracts served as positive control samples, and 200-fold excess wild-type and mutated NF-κB consensus oligonucleotides were used to test the specificity of the assay.

NF-κB (p65) Immunohistochemistry

Paraffin-embedded endometrial tissue was cut into semiserial sections of 5 μm. Immunohistochemistry was done with the Histostain SP-Kit (AEC) (Invitrogen,) as previously described (13). Mouse monoclonal antibody to the human NF-κB p65 (RelA) subunit (Santa Cruz Biotechnology) was used at a 1:100 dilution overnight at 4°C. Negative control samples were carried out in the absence of primary antibody and in the presence of immunoglobulin G1 (normal mouse IgG1; Santa Cruz Biotechnology).

The expression level score (ELS), calculated by means of Image Pro Plus software (Media Cybernetics), measured the nuclear and cytoplasmic expression of p65 in the total epithelial or stromal surface area of a single section of each endometrial sample.

Statistical Analyses

Analyses of p65-DNA binding are presented as values relative to the positive control sample. Mean values ± SE are given in the Results section, and box plot graphics are shown in the figures. One-way analysis of variance followed by Tukey post hoc test was used to analyze results comparing the three different phases of the menstrual cycle. The Mann-Whitney U test was used to compare results between endometriosis patients and control subjects during the same menstrual cycle phase. The Wilcoxon signed-rank test was used to compare paired groups between endometrial epithelial and stromal cells on the same immunostained slides. Statistical analyses were performed using SPSS software. A P value of $<.05$ was considered to be statistically significant. All experiments were done in duplicate.

RESULTS
Total NF-κB–DNA Binding in Human Endometrium

EMSA analyses showed variable constitutive NF-κB–DNA binding in human endometrium of healthy women and endometriosis patients (Fig. 1). NF-κB–DNA binding activity was present in all phases of the menstrual cycle in healthy women and in endometriosis patients, being strongly variable. Some samples showed strong NF-κB–DNA binding and others very low or undetectable levels. Figure 1 depicts two characteristic EMSAs showing NF-κB–DNA binding in menstrual endometrial nuclear extracts from healthy women (Fig. 1A) and endometriosis patients (Fig. 1B).

NF-κB (p65)–DNA Binding Immunodetection in Human Endometrium

DNA binding of the p65 subunit of NF-κB (Fig. 2) exhibited higher levels in proliferative endometrium than in menstrual and secretory endometrium of healthy women (1.02 ± 0.14, 0.61 ± 0.1, and 0.62 ± 0.08, respectively; $P<.05$). In endometriosis patients, p65-DNA binding was lower in menstrual endometrium than in proliferative and secretory endometrium (0.42 ± 0.03, 0.83 ± 0.09, and 0.88 ± 0.1, respectively; $P<.05$). No significant differences were observed between endometrial control subjects and the respective cycle-phase endometriosis patients.

NF-κB (p65) Immunolocalization and Expression

Immunohistocehmistry showed p65 expression in human endometrium in all phases of the menstrual cycle in both endometrial epithelial cells (EEC) and endometrial stromal cells (ESC). p65 was immunolocalized in the cytoplasm and in some nuclei of endometrial cells (Fig. 3).

The ELS of p65 was higher in EEC than in ESC ($P<.05$), and no significant differences were observed between the different phases of the menstrual cycle in control subjects and endometriosis patients (Fig. 3).

DISCUSSION

Acknowledging peritoneal endometriosis as a disease originating from refluxed endometrial tissue (40–43), numerous
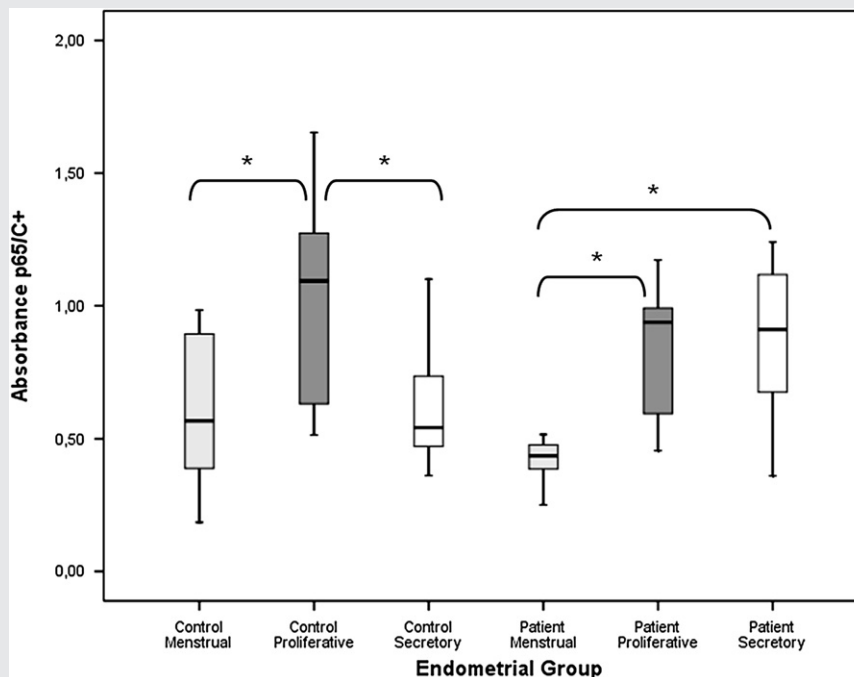

FIGURE 1
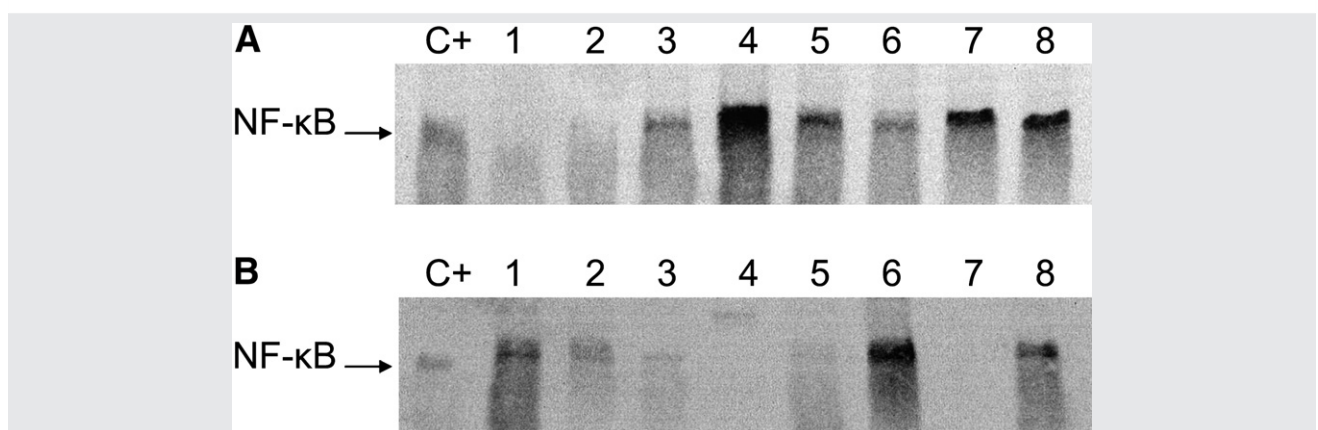
Electrophoretic mobility shift assay of nuclear factor (NF) κB. (A) Healthy women endometrial samples. (B) Endometriosis patients endometrial samples. Lanes 1–8 = nuclear extracts of endometrial samples from different patients; C+ = positive control sample.

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FIGURE 2

p65-DNA binding immunodetection assay. The absorbance of the p65 protein–oligonucleotide immunodetected complexes was measured relative to positive control sample (C+). Control endometrial samples were obtained from healthy women, and patient endometrial samples were obtained from endometriosis patients. Box plots show the median, 25th and 75th percentiles, and range. *$P < .05$.

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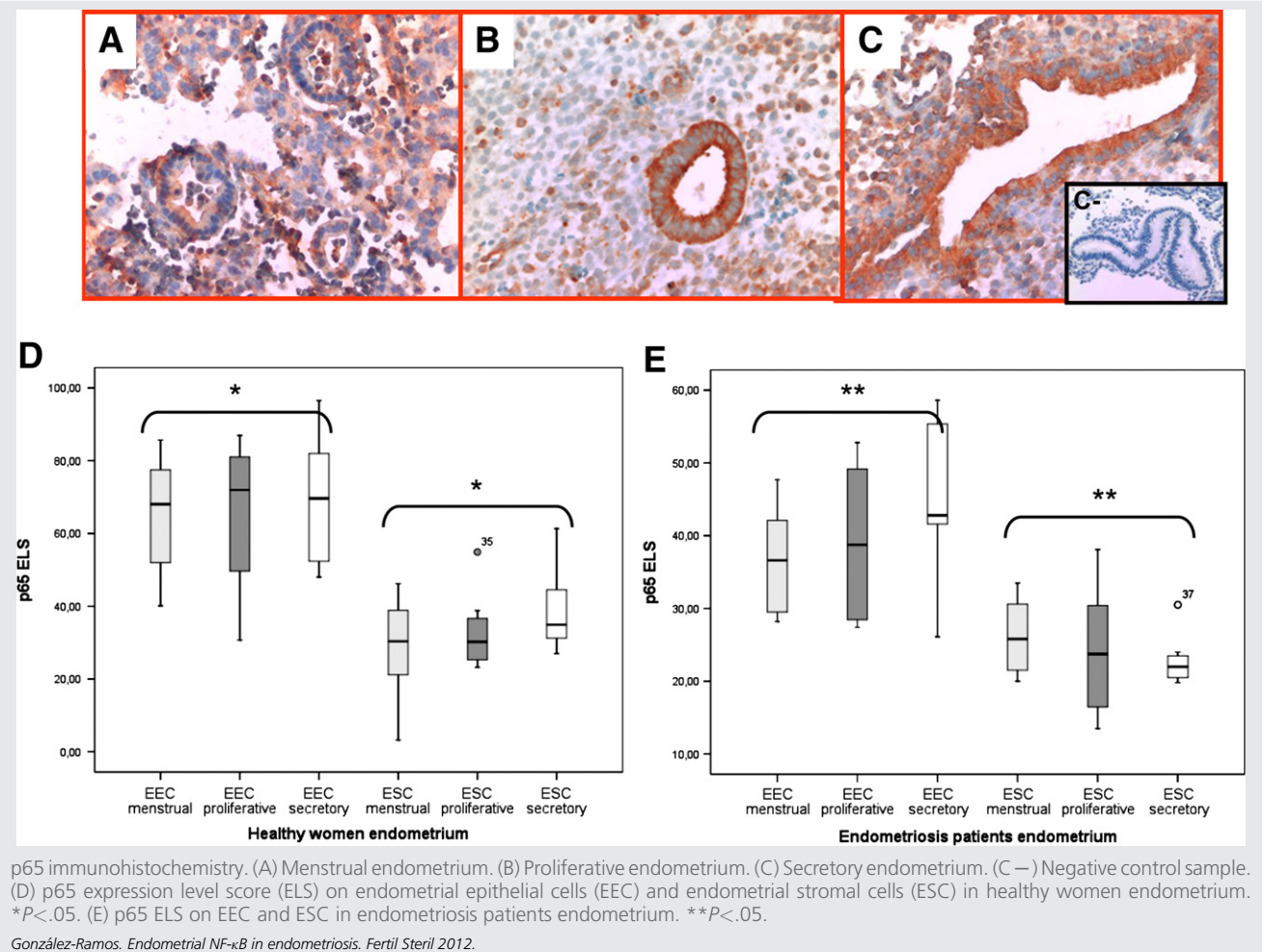
researchers have studied eutopic endometrium under physiologic conditions, comparing it with eutopic endometrium of endometriosis patients. Thus, many biomolecular differences between normal endometrium and endometriosis endometrium have been demonstrated (43–45). Along this line, our study investigated human endometrial tissue from healthy women and endometriosis patients, considering the three main phases of the menstrual cycle.

In the introduction, we described the main results and conclusions regarding NF-κB signaling in endometriosis development, and three reviews on this subject have been published recently (11, 15, 16). Most of the studies involving NF-κB in endometriosis pathophysiology are in vitro or animal in vivo investigations (13, 21–33). Others have studied NF-κB–DNA binding in human endometrial or endometriotic tissues in vivo (12, 37). The present study evaluated in vivo endometrial NF-κB activation in a determined moment, and consequently it must be interpreted as a biologic instant of the endometrium in the studied women. It is also important to consider that the classic NF-κB pathway reacts within minutes (4–6), and therefore the NF-κB activation we observed is not a constant phenomenon. Also, the laboratory techniques used in this study are complementary, contributing different types of information. Thus, EMSA showed total NF-κB–DNA binding, including different NF-κB dimers, the immuno-DNA binding assay showed only p65-DNA binding, and immunohistochemistry showed p65 expression and localization in endometrial cells but not p65-DNA binding.

Physiologic activity of NF-κB in proliferative and secretory endometrium was demonstrated qualitatively in an earlier study using EMSA (37). Here, we complement that information by adding menstrual endometrial samples and by quantitatively evaluating p65 activity using the p65-DNA binding immunodetection assay.

Little is known about endometrial NF-κB and hormonal regulation in vivo. The present study is the first contributing some information on physiologic cyclic regulation of NF-κB activity in women's endometrium across the menstrual cycle. Importantly, this study analyzed DNA binding of p65, one of the five NF-κB subunits (p65 or RelA, p50/p105, p52/p100, RelB, and c-Rel) and which has been shown to be activated by the classic and atypical NF-κB pathways. The cyclic p65-DNA binding pattern observed in this group of healthy women (increased binding in proliferative endometrium versus menstrual and secretory endometrium) is in agreement with works demonstrating NF-κB inhibition by progesterone (P) or progestational compounds in endometriotic stromal cells (27). Additionally, negative interaction between p65 and P receptor in other cell types (46) and antagonistic crosstalk with estrogen receptors (ER) in ESC and other cell types have been described (47–52). Other authors had proposed that P inhibition of NF-κB activity during the secretory phase could be suppressed by P withdrawal, thereby increasing NF-κB activation during menstruation; but none of them directly and/or quantitatively studied NF-κB–DNA binding in menstrual endometrial tissue (17, 35).

FIGURE 3

p65 immunohistochemistry. (A) Menstrual endometrium. (B) Proliferative endometrium. (C) Secretory endometrium. (C −) Negative control sample. (D) p65 expression level score (ELS) on endometrial epithelial cells (EEC) and endometrial stromal cells (ESC) in healthy women endometrium. *P<.05. (E) p65 ELS on EEC and ESC in endometriosis patients endometrium. **P<.05.

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Our results did not show increased p65-DNA binding in menstrual endometrium. However, other non–p65-NF-κB dimers, not assessed in this study, could bind to DNA during menstruation, as postulated by other authors. Transcriptional activity of p65-containing dimers could stimulate proliferative and antiapoptotic processes of endometrial cells during the proliferative phase of the menstrual cycle, as it has been shown in other studies (13, 30). As well, p65-containig dimers might positively regulate physiologic innate immunity processes of the endometrium after menstruation. Since p65-DNA binding was increased during the proliferative phase, estrogens may not be inhibiting p65 activity in the endometrium or could be a stimulating factor of p65 activity. NF-κB activity is modulated in a complex manner and by many stimuli, not only by hormones and/or hormonal receptors, and this study does not allow us to answer accurately on how hormones and hormonal receptors regulate NF-κB activity. The reduced p65-DNA binding in menstrual and secretory endometrium of healthy women suggests that p65 activity may not be an important modulator of cell processes in these phases of the menstrual cycle or that basal p65 activity is enough to activate transcription of mediators involved in

physiological immunological and cell survival processes as well to modulate transcription of proteins involved in menstruation and/or implantation.

Our results demonstrate endometrial modifications in this group of endometriosis patients. The physiologic pattern of endometrial NF-κB activation was lost in endometriosis patients, showing similar high p65 activity levels in proliferative and secretory endometrium of endometriosis patients. We hypothesize that this response could be explained by diverse degrees of P insensitivity in endometrial cells of endometriosis patients, as has been stated in recent studies (53–57).

Increased endometrial p65 activity during the proliferative and secretory phases of the menstrual cycle in endometriosis patients reveals a longer period of p65 activity in the endometrium of endometriosis patients compared with the normal endometrium of healthy women. This pattern could also be present in refluxed endometrial cells after menstruation, enhancing proliferative, antiapoptotic, and proinflammatory processes in these ectopic cells and favoring peritoneal endometriosis establishment and development. These premises are sustained by earlier works demonstrating positive modulation of proliferative, antiapoptotic, and proinflammatory actions

by NF-κB in endometrial and endometriotic cells (13, 19–33). Furthermore, the increased p65 activity in secretory endometrium could influence implantation dysfunctions and thus endometriosis-associated infertility.

Immunohistochemistries showed higher p65 expression in the epithelial compartment than in the stromal compartment of the endometrium, which could reflect a more important role of this protein in epithelial cell functions than in stromal cells.

In conclusion, this study demonstrates constitutive and physiologic NF-κB–DNA binding in human endometrium with increased p65-DNA binding in proliferative endometrium under physiologic conditions. Decreased physiologic p65 activity in secretory endometrium may be a consequence of P action. The role of NF-κB and its different activation pathways and dimers in the endometrial physiology remains to be elucidated, but according to its known functions, NF-κB could modulate inflammation, immunology, cell survival, adhesion, invasion, and angiogenesis in the endometrium. In endometriosis patients' endometrium, p65-DNA binding was shown to be altered during the menstrual cycle, revealing diminished cyclic variations with increased activity in proliferative and secretory endometrium, which could be involved in endometrial dysfunctions of endometriosis patients and in endometriosis pathophysiology.

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