Iron overload–modulated nuclear factor kappa-B activation in human endometrial stromal cells as a mechanism postulated in endometriosis pathogenesis

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Objective: To evaluate the effect of iron overload on nuclear factor kappa-B (NF-κB) activation in human endometrial stromal cells (ESCs).
Design: Experimental study.
Setting: University hospital research laboratory.
Patient(s): Ten healthy women.
Intervention(s): Isolated ESCs from endometrial biopsies were incubated with 50 μM $FeSO_4$ or vehicle. The NF-κB inhibitor [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide (TPCA-1), which inhibits IKKβ, the kinase of IκBα (inhibitory protein of NF-κB), was used to prevent iron overload–stimulated NF-κB changes in ESCs.
Main Outcome Measure(s): NF-κB activation was assessed by p65:DNA-binding activity immunodetection assay. IκBα, p65, and intercellular adhesion molecule (ICAM)-1 proteins expression was evaluated by Western blots. ESC soluble ICAM (sICAM)-1 secretion was measured by ELISA using conditioned medium.
Result(s): Iron overload increased p65:DNA-binding activity and decreased IκBα and p65 cytoplasmic expression in ESCs after 30 minutes of incubation as compared with the basal condition. ESC ICAM-1 expression and sICAM-1 secretion were higher after 24 hours of iron overload treatment than in the absence of treatment. TPCA-1 prevented the iron overload–induced increase of p65:DNA binding and IκBα degradation.
Conclusion(s): Iron overload activates IKKβ in ESCs, stimulating the NF-κB pathway and increasing ICAM-1 expression and sICAM-1 secretion. These results suggest that iron overload induces a proendometriotic phenotype on healthy ESCs, which could participate in endometriosis pathogenesis and development. (Fertil Steril® 2015;103:439–47. ©2015 by American Society for Reproductive Medicine.)
Key Words: Endometriosis, endometrium, iron overload, NF-κB, ICAM-1

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The local environment of the peritoneal cavity of women with endometriosis has been associated with an inflammatory reaction. In this context, the transcription factor nuclear factor kappa-B (NF-κB) has been involved in the stimulation of inflammation and cell survival of ectopic endometrial cells, promoting cell proliferation and inhibiting apoptosis (1–3). The NF-κB activation

pathways are complex and have been described in the context of endometriosis in a recent review (4). Briefly, p65/p50 dimers of NF-κB are activated by proinflammatory cytokines and oxidative stress, between other stimuli, which trigger IκB-alpha (IκBα) phosphorylation by the kinase beta of IκB (IKKβ) or tyrosine kinase and the casein kinase II. IκBα phosphorylation is followed by its ubiquitination and degradation. Thus, p65/p50 free dimers translocate to the cell nucleus, where they bind to DNA, activating the transcription of genes containing κB sequences in their promoters. This type of NF-κB activation is fast acting and occurs within minutes (5–10). The binding of p65/p50 dimers to DNA results in the transcription of hundred of genes involved in inflammation, adhesion, invasion, angiogenesis, cell proliferation, and apoptosis (11, 12). Among these genes, the intercellular adhesion molecule 1 (ICAM-1 or CD54) works mainly as a counter-receptor for the lymphocyte function–associated antigen 1 (LFA-1), a kind of integrin present in leukocytes. It can participate in many inflammatory-related events (i.e., cell interactions, chemotaxis, and proinflammatory responses), since stimuli like interleukin (IL)-1, tumor necrosis factor (TNF)-α, and angiotensin II can modulate ICAM-1 levels and the release of its soluble form, sICAM-1, which can attach to LFA-1 and interrupt the interaction between leukocytes and ICAM-1 expressing cells (13–15). ICAM-1 protein or mRNA overexpressions have been documented in serum, peritoneal fluid, and ectopic endometrial stromal cells of women with endometriosis, which suggests a role of this molecule in the pathophysiology of endometriosis (16–19).

The lysis of erythrocytes swept along to the peritoneal cavity by retrograde menstruation produces iron release (20). In fact, many studies have shown iron overload in the peritoneal fluid, peritoneal macrophages, and endometriotic tissue of women with endometriosis, implicating iron overload in the pathogenesis of the disease (21–24). Iron is a vital oligoelement, but in supraphysiological quantities it can have adverse effects on the cells, because it reacts with metabolism-related molecules as hydrogen peroxide and superoxide anion, favoring the formation of highly toxic hydroxyl radicals and then the establishment of an oxidative setting (25, 26). Iron overload activates NF-κB in hepatic macrophages, human prostate cancer, and lung and colorectal adenocarcinoma cell lines (27–31), and in vitro studies carried out with the Caco-2 cell line and endothelial cells indicate that iron, in an NF-κB-dependent response, can promote a rise of ICAM-1 levels (32, 33). Until now there are no data about these bonds in endometrial cells. Using endometrial stromal cells (ESCs) from healthy women as a working model, we aimed to ascertain whether iron overload can act as an NF-κB activation inductor and promote a proendometriotic inflammatory response in these cells.

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) during the proliferative phase (days 5–13) of the menstrual cycle from 10 healthy women (age, 26–39 years; mean, 32.6 ± 4.0 years) who were not receiving hormone treatment and who were undergoing laparoscopic surgery for tubal sterilization. The endometrial phase of the biopsies was histologically confirmed according to the standard criteria described by Noyes and collaborators (34). The samples were immediately placed on ice, transported, and cleaned of blood excess and clots with sterile phosphate-buffered saline (PBS; Gibco). A portion of each sample was fixed in 4% buffered formaldehyde and embedded in paraffin for dating. Another endometrial portion was used for ESC isolation procedures.

Cell Culture and Stimulation

The endometrial tissue was gently minced into small pieces with scalpels until it reached a paste-like appearance. Then minced tissue was incubated for 1 hour at 37°C in a shaking water bath in 2 mL phenol red-free Dulbecco's modified Eagle's medium (DMEM/F12-prf) (Gibco) containing collagenase type VII (1 mg/mL; Sigma) and bovine pancreas DNase-I (10 μg/mL; Sigma). Then the dissociated cells were gently filtered through 40- and 100-μm wire sieves. ESCs were further purified from the remaining epithelial cells by selective adherence, plated in T-75 flasks (Orange Scientific), and allowed to adhere for 20 minutes (35). Supernatant medium containing erythrocytes, epithelial cells, and debris was then removed and replaced by fresh DMEM/F12-prf containing 10% vol/vol fetal bovine serum (FBS; Biological Industries) and 1% vol/vol antibiotics/antimycotic (Gibco). All cultures were maintained at 37°C and 5% CO_2 in a humidified chamber (model 3164, Forma Scientific Inc.). The purity of obtained ESCs was assayed by immunocytochemistry with antibodies to vimentin (stromal cell marker) and cytokeratin-18 (epithelial cell marker; EMD Millipore) as described elsewhere (35). ESC cultures contained no detectable cytokeratin-positive cells. Whenever necessary, ESCs were suspended in medium with 10% vol/vol dimethyl sulfoxide (DMSO; Sigma) and stored in liquid nitrogen. For stimulation, cells from the second passage were plated in 100-mm Petri dishes (Orange Scientific) until they reached 80% confluence. Then the medium was discarded and replaced for 18–24 hours by FBS-free medium. The next day, the plates were washed with PBS, the FBS-free medium was replaced, and ESCs were stimulated with $FeSO_4 \cdot 7H_2O$ (Sigma) at 50 μM (17.5 μL from freshly 20 mM stock solution in 7 mL FBS-free medium) during incubation periods of 30 minutes and 2, 6, and 24 hours. Control condition (iron-free) corresponds to ESCs coming from the same biopsy but cultured with FBS-free medium alone. The iron overload concentration used in these experiments corresponds to the iron concentration measured in the peritoneal fluid from patients with endometriosis (24), and it is the same that was used in previous studies in other cell types (27–30). Additionally, ESCs from one biopsy were stimulated with 1 ng/mL IL-1β (Sigma) at 30 minutes and 2, 6, and 24 hours as positive controls for NF-κB pathway activation (36–38).