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).

Cell viability was determined by reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) and by measurement of extracellular lactate dehydrogenase activity, using the LDH-Cytotoxicity Assay Kit II (BioVision). Using 96-well plates, 1.5×10^4 ESCs/well were seeded five times, serum deprived for 18–24 hours, and stimulated for 24 hours with 50 μM $FeSO_4$. Following the manufacturer's instructions, optical density measurement at 490 and 655 nm (reference wavelength) was performed using a microplate reader (PHOmo spectrophotometer; Autobio Labtec Instruments). Both assays were carried out twice and independently. The 24-hour exposure to iron overload did not affect ESC viability (data not shown).

NF-κB Inhibition

Studies on hepatic macrophages suggest that iron overload triggers the pathway by activating IKKβ (27, 28, 39), so we used the IKKβ inhibitor TPCA-1 ([5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide; Santa Cruz Biotechnology) at a 10-μM concentration. This concentration has been demonstrated to be effective in primary human trophoblast cells and choriodecidual cell cultures (40–42). The inhibitor vehicle was 0.01% vol/vol DMSO. DMSO did not induce any variation on IκBα or p65 cytoplasmic protein expression and had no influence on p65:DNA complexes formation (data not shown). The inhibitor was added to cell cultures 45 minutes before the 30-minute stimulus with $FeSO_4$ or IL-1β.

Extraction of Cytoplasmic and Nuclear Proteins

After the exposure to $FeSO_4$ or control experiments, in the absence or presence of TPCA-1, cells were trypsinized, collected, and centrifuged for 5 minutes at $400 \times g$. The pellet was resuspended in 500 μL PBS, placed in a 1.5-mL tube, and centrifuged again at $400 \times g$ at 4°C for 5 minutes. Proteins from pelleted cells were extracted using nuclear and cytoplasmic extraction reagents (NE-PER, Pierce) according to the manufacturer's protocol. Protease inhibitor cocktail (Sigma) and phosphatase inhibitors, 500 μM sodium orthovanadate (Sigma) and 1 mM sodium fluoride (Sigma), were added to the extraction reagents. Cytoplasmic and nuclear extracts were stored at −80°C until use. Protein concentrations were determined using the BCA protein assay kit (Pierce). Sample freezing did not alter protein concentrations or quality, as established in preliminary experiments.

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, as described elsewhere (43). 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 and a p65 monoclonal antibody, which specifically recognizes an epitope that is only exposed when protein is activated, was added to each well; horseradish peroxidase–conjugated secondary antibody was used to amplify the signal. After adding developing solution, the absorbance at 450 nm with a reference wavelength of 655 nm was examined on a microplate reader. Samples were tested in duplicate. Specificity of the assay was evaluated using controls provided by the manufacturer in duplicate.

Western Blots

Protein expression of IκBα, p65, and ICAM-1 was analyzed in cytoplasmic extracts. Protein extracts (20 μg per lane) were heated at 95°C for 5 minutes and then resolved by 12% vol/vol sodium dodecylsulphate-polyacrilamide gel electrophoresis and transferred onto nitrocellulose membranes (0.45 μm; Pierce) for 1.5 hours at constant 350 mA. After Ponceau red (Sigma) staining to check proper transfer, membranes were blocked with 5% wt/vol dry nonfat skimmed milk powder in 0.1% vol/vol Tween-20/Tris-buffered saline (TBST) at pH 7.5 for 2 hours of shaking at room temperature (RT). The membranes were cut, and different sections were then incubated overnight at 4°C with IκBα (sc-371), p65 (sc-372), or ICAM-1 (sc-7891) rabbit polyclonal primary antibodies (Santa Cruz Biotechnology), which were used at 1:750, 1:2,000, and 1:1,000 dilutions, respectively. Membranes were incubated with anti-β-actin mouse monoclonal antibody (clone AC-15, Sigma) diluted at 1:8,500 as an internal control for protein loading and transfer. Additional incubations with rabbit and mouse IgG (Santa Cruz Biotechnology) were done as negative controls. All antibodies were prepared in 1% blocking solution. After washes with TBST, membranes were incubated for 1 hour at RT and agitated with horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch) diluted 1:8,500 in TBST. Protein detection was achieved using Western Lightning Enhanced Chemiluminiscent Reagent Plus (PerkinElmer). Chemiluminescence was captured with a CCD digital camera (Discovery 10gD, Ultralüm), and the optic density of the IκBα, p65, and ICAM-1 bands relative to the β-actin bands was measured and analyzed using computational software (ImageJ 1.42q; National Institutes of Health). Samples were tested in duplicate.

Soluble ICAM-1 ELISA

Soluble ICAM-1 was quantified in cell-free conditioned medium. Once collected, supernatants were centrifuged for 5 minutes at $400 \times g$ and then aliquoted and stored at −80°C until use. Protein levels were evaluated using the ELISA kit Quantikine for human sICAM-1/CD54 (R&D Systems) following the manufacturer's instructions. In this assay, 1 mL of each cell-free conditioned medium was concentrated 5 times (200 μL final volume) by evaporation during 2 hours at 30°C with a concentrator device (Concentrator 5301; Eppendorf). Next, 100 μL were put into the wells, and after the corresponding incubations and the addition of developing solution, the absorbance at 450 nm with a reference wavelength of 655 nm was examined on the spectrophotometer.

The concentration of sICAM-1 was extrapolated from a standard curve using recombinant sICAM-1. Values were normalized to the protein content of each culture from which the conditioned medium was obtained. Samples were tested in duplicate. The limit of detection of the kit was 96 pg/mL, and the inter- and intra-assay coefficients of variation were 4.6% and 5.5%, respectively.

Statistical Analysis

Data are expressed as the mean of five independent experiments coming from five different endometrial biopsies ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's test was performed for multiple comparisons between control conditions and each stimulus. For experiments with TPCA-1 inhibitor and comparisons between conditions, a Bonferroni post-test was performed. $P<.05$ was considered statistically significant. All analyses were performed using the computational software Prism v5.01 (GraphPad Software Inc).

RESULTS

Effect of Iron Overload on Cytoplasmic NF-κB Pathway Components

Iron overload decreased IκBα expression 0.5-fold versus control conditions at 30 minutes and 2 hours of incubation time ($P<.01$; Fig. 1A and B). In the case of p65, iron overload induced a reduction (0.4-fold; $P<.05$) of cytoplasmic p65 expression at 30 minutes' exposure, while no significant change was observed at 2 hours' incubation time, as compared with the control condition (Fig. 1A and C). IL-1β reduced IκBα and p65 cytoplasmic expression in ESC, showing a similar response to the iron overload stimulus (data not shown).

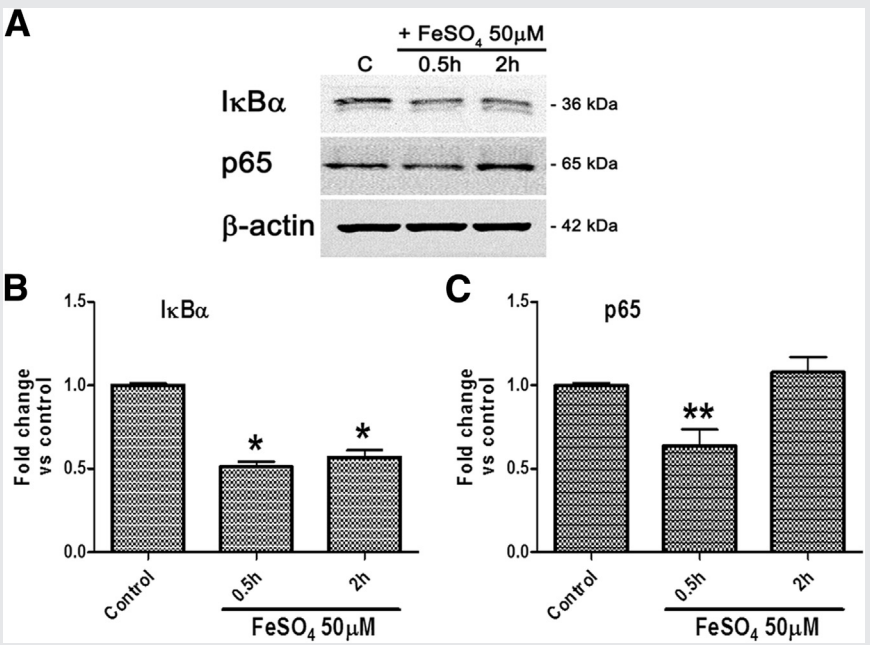
Effect of Iron Overload on p65 Binding to DNA

At 30 minutes of stimulus with $FeSO_4$ 50 μM, an increase (1.4-fold, $P<.05$) in p65:DNA interaction was shown versus control cultures, whereas at 2 hours of treatment there was no change relative to the control experiment, indicating a short acting time of the pathway (Fig. 2A). The specificity of the assay was confirmed using nuclear extracts from Jurkat cells stimulated with phorbol ester as a positive control for p65:DNA complexes. In the presence of wild-type soluble κB oligonucleotides there was a clear reduction in p65 binding to DNA, while using mutated oligonucleotides did not interfere with the formation of p65:DNA complexes (Fig. 2B). In a similar way to iron overload stimulus, IL-1β induced an increase in p65:DNA binding in ESCs (data not shown).

Effect of Iron Overload on ICAM-1 Expression

To determine the downstream effect of iron overload–dependent NF-κB activation, the NF-κB-modulated protein ICAM-1 was assessed by Western blot using cytoplasmic extracts of ESCs, and sICAM-1 was quantified in culture media by ELISA. It is shown that after 24 hours of stimulus with $FeSO_4$ 50 μM, there is an increase in protein expression (4.8-fold vs. control; $P<.05$; Fig. 3A and B). At 24 hours of treatment

FIGURE 1



IκBα and p65 expression in response to iron overload in ESC. (**A**) Western blot picture of IκBα, p65, and β-actin proteins using ESC cytoplasmic extracts after 0.5 and 2 hours of exposure to iron overload. C = control condition. Protein weight is indicated in kilo-Daltons (kDa) (**B**) IκBα expression relative to β-actin bands. (**C**) p65 expression relative to β-actin bands. The bars show the mean ± SEM of five independent values (n = 5). Statistics are performed with one-way ANOVA with Dunnett's test (*$P<.01$ and **$P<.05$ vs. control condition).

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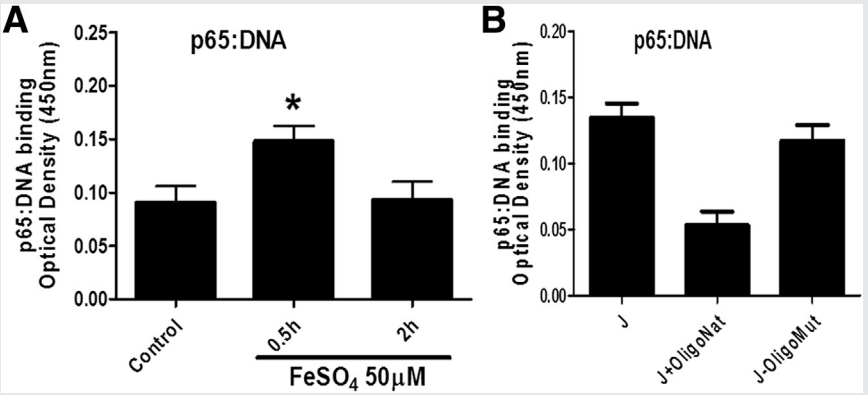
FIGURE 2



p65:DNA binding in response to iron overload in ESC. (**A**) Immunodetection assay for p65:DNA complexes (TransAM). The bars show the mean ± SEM of five independent values (n = 5) expressed as optical density measured at 450 nm. (**B**) The specificity of the assay was evaluated as described in Materials and Methods. J = Jurkat cells nuclear extracts; OligoNat = wild type κB oligonucleotide; OligoMut = mutated κB oligonucleotide.Statistics are performed with one-way ANOVA with Dunnett's test (*P<.05 vs. control condition).

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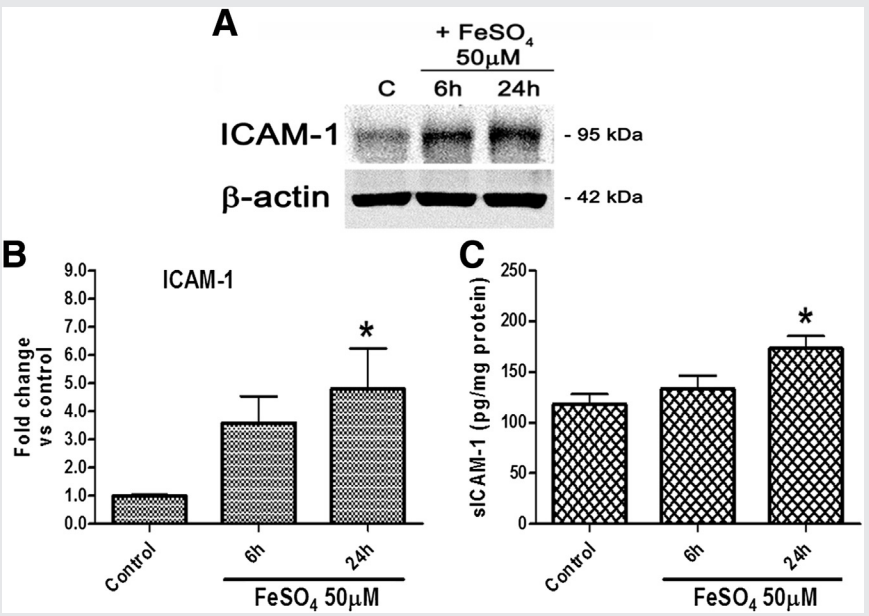
with iron overload, ESCs increased the secretion of sICAM-1 to the extracellular medium (1.4-fold vs. control; P<.05; Fig. 3C). IL-1β induced an increase of ICAM-1 expression in ESCs, similar to iron overload stimulus (data not shown).

NF-κB Inhibition Experiments

Pretreatment for 45 minutes with TPCA-1 of ESCs before incubation with $FeSO_4$ 50 μM for 30 minutes effectively blocked the iron overload–induced reduction of cytoplasmic IκBα expression, showing a 1.3-fold increase versus the iron overload condition (P<.05). In the TPCA-1-alone condition there was a significant increase of cytoplasmic IκBα expression versus the control condition (1.5-fold; P<.001; Fig. 4A and B). Preincubation with TPCA-1 increased 1.4-fold the expression of p65 versus the vehicle condition (P<.05; Fig. 4A and C). Pretreatment with TPCA-1 prevented the effect of iron overload on p65:DNA binding and induced a

FIGURE 3



ICAM-1 expression in ESC and sICAM-1 levels in culture media of ESCs. (**A**) Western blot picture of ICAM-1 and β-actin using cytoplasmic extracts from ESCs. C = control condition. Protein weight is indicated in kilo-Daltons (kDa). (**B**) ICAM-1 expression relative to β-actin bands. (**C**) sICAM-1 ELISA using ESC conditioned medium. The bars show the mean concentration (pg/mg protein) ± SEM of five independent values (n = 5). Statistics are performed with one-way ANOVA with Dunnett's test (*P<.05 vs. control condition).

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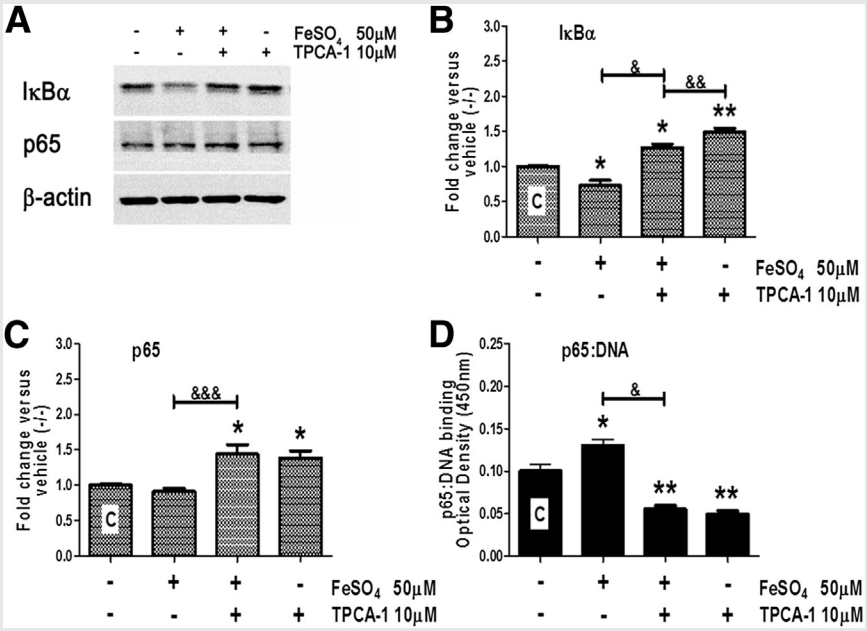
FIGURE 4



Effect of IKKβ inhibition before iron overload exposure in ESCs on IκBα and p65 cytoplasmic expression and p65:DNA binding. (**A**) Western blot picture of IκBα, p65, and β-actin proteins using ESC cytoplasmic extracts. (**B**) IκBα expression relative to β-actin bands. (**C**) p65 expression relative to β-actin bands. (**D**) Immunodetection assay for p65:DNA complexes (TransAM). The bars show the mean ± SEM of five independent values (n = 5) expressed as optical density measured at 450 nm. C = control condition (vehicle alone). Statistics are performed with one-way ANOVA followed by the Bonferroni post-test (*P<.05 and **P<.001 vs. vehicle; $^{\&}P$<.001, $^{\&\&}P$<.05, and $^{\&\&\&}P$<.01 between conditions).

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reduction (0.5-fold vs. vehicle; P<.001) of p65:DNA interaction. This response was similar to the inhibitor-alone condition (0.5-fold vs. vehicle; P<.001; Fig. 4D). Thus, TPCA-1 prevented iron overload–stimulated NF-κB activity and basal ESC NF-κB activity. In control experiments, TPCA-1 inhibited IL-1β-induced IκBα degradation and p65 cytoplasmic expression reduction in ESCs (data not shown) in the same manner as in iron overload experiments.

DISCUSSION

In patients with endometriosis, iron overload has been shown in the peritoneal tissue, liquid, and macrophages and in the eutopic and ectopic endometrium (21–24). Iron excess acts as a pro-oxidant factor and inductor of proinflammatory pathways in macrophages and human lung and colorectal adenocarcinoma cells (27, 28, 30, 31). On the other side, NF-κB is constitutively activated in endometriotic lesions (44); inhibition of NF-κB in in vitro and in vivo animal models of the disease has shown a reduction of endometriotic lesion development, decreasing inflammation and cell proliferation, and increasing apoptosis of endometriotic cells (45, 46). In the endometrium, NF-κB is physiologically activated, but the cyclic activation pattern is altered in women with endometriosis (43), and previous review studies have postulated iron overload as an inducer of NF-κB activation in endometrial or endometriotic cells as a mechanism involved in endometriosis pathophysiology (47, 48). Our results show that iron overload induces NF-κB pathway activation in healthy human ESCs, which is reflected by events at 30 minutes of stimulus, like IκBα degradation, reduction of cytoplasmic p65, and its union to oligonucleotides with κB consensus sequence. Furthermore, the iron overload effect would involve at least partially the mediation of IKKβ activity, as suggested by the experiments in which the IKKβ inhibitor, TPCA-1, prevented or reversed the iron overload–induced changes in ESC, that is, inhibiting IκBα degradation and p65:DNA binding. It is known that NF-κB activation by IL-1β involves IKKβ activity (8, 49, 50), and our control experiments showed similar results in response to IL-1β or iron overload in ESCs in the presence or absence of TPCA-1, which also supports IKKβ as the main kinase activating the NF-κB pathway in response to iron overload in ESCs. Iron proinflammatory signaling has been suggested to be dependent mainly on pro-oxidative hydroxyl ($^{\bullet}$OH) and peroxynitrite ($ONOO^{-}$) radical formation, which would promote the activity of IKKβ (27, 28, 51). This type of specific cell signaling, linking iron overload and p65 activation by IKKβ, was previously suggested to be exclusive of hepatic macrophages (28, 39). Several studies indicate that iron can promote p65:DNA complexes formation, but no data about IκBα degradation dynamics or IKKβ involvement are provided (29–31). This study shows that this type of cell signaling, involving iron overload–mediated IKKβ and p65 activation, is also present in ESCs and not only in hepatic macrophages. However, these cell types exhibit some differences in NF- κB signaling kinetics. Our results show that iron overload induces an early and short effect on NF- κB activation that returns to the basal

level at 2 hours with no recovery of IκBα cytoplasmic levels. On the other hand, hepatic macrophages exhibited NF-κB activation at 30 minutes and 2 hours as well, and certain IκBα recovery was seen at 2 hours poststimulus (27). Beyond evident cell type–specific differences, there are some molecular mechanisms to be considered as possible modulators of these observations. Cessation of NF-κB activity occurs mainly owing to upregulation of IκB proteins (2, 9, 10). Studies in mouse embryonic fibroblast, HeLa, and Jurkat cells showed that NF-κB can induce an early expression (within 1 hour and delayed by 45 minutes with respect to that of IκBα) of IκBε, which acts as effectively as IκBα on the inhibition of p65:DNA interaction (11, 52, 53). Alternatively, NF-κB can induce the synthesis of the Sef protein, which was recently identified as a feedback antagonist of receptor tyrosine kinase signaling. Overexpression of Sef suppressed IL-1-induced IKKβ dependent p65/p50 NF-κB dimers activation. This response was associated with the inhibition of NF-κB -dependent de novo synthesis of the IκBα protein (54). Sef overexpression reduces endometrial adenocarcinoma cell proliferation, and loss of Sef expression has been shown in the endometrium of women with adenomyosis (55, 56). Finally, oxidative stress can promote IκBα phosphorylation by casein kinase II and Syk tyrosine-kinase, favoring its degradation (57–59). Although attractive as explanations, these mechanisms have not been demonstrated to operate in endometrial cells, and no direct evidence of iron overload participation is available. Further studies should be carried out to unravel the mechanisms that govern precise iron overload signaling in ESC. The statistically significant 1.5-fold increase in NF-κB:DNA binding, observed after 30 minutes of iron overload stimulus, may seem modest, but it agrees with responses shown in other cellular systems. For instance, studies on neuroblastoma, cerebral endothelial, and lung adenocarcinoma cells, using higher iron concentrations (77–150 μM) and equal or longer times of stimulus (30 minutes–24 hours), have consistently shown that, compared with control condition, iron overload induces just a 50% mean increase on NF-κB activation, which appears to be sufficient to trigger long-term and important cellular responses, such as the expression of inducible nitric oxide synthase 2 and the release of IL-8 and macrophage chemotactic protein-1 to the extracellular medium (31, 60, 61).

As a consequence of the iron overload stimulus, ICAM-1 expression in ESC and sICAM-1 secretion by ESC were significantly increased after 24 hours of exposure time, which is most probably due to the effect of NF-κB activation at a short time after iron overload stimulus, since ICAM-1 is known to be modulated by NF-κB (13, 32, 33). The statistically significant 4.8-fold increase in ICAM-1 expression and 1.4-fold increase in sICAM-1 secretion by ESCs in response to iron overload seem biologically relevant, but further experiments should be carried out to test biological cellular changes. ICAM-1 expression by refluxed endometrial cells during menstruation may allow their adhesion to the mesothelial lining. Besides, as T-cytotoxic lymphocytes and natural killer cells express ICAM-1 ligands like LFA-1 and Mac-1 proteins, it has been suggested that sICAM-1 release could impede the interaction between immune system cells and refluxed ones, thus avoiding ectopic endometrial cells elimination (16–19).

Retrograde menstruation may occur in up to 90% of women of reproductive age at some moment of their lives, but only 5%–10% develop endometriosis. Nevertheless, intense and long-lasting menstrual fluxes, as well as shorter menstrual cycles, are known risk factors in patients with endometriosis, which clearly produce more exposure to menstrual reflux, supporting Sampson's theory (62–66). Intrapelvic iron overload (including peritoneal liquid, endometriotic lesions, and macrophages), the most probable and logical source of which are refluxed erythrocytes during menstruation and the bleeding of endometriotic lesions themselves, has been shown in patients with endometriosis as opposed to in healthy women (20–24). Thus, iron overload emerges as an important etiological factor. In most women, refluxed endometrial tissue is eliminated from the peritoneal cavity by peritoneal macrophages, but apparently this mechanism becomes dysfunctional in women with endometriosis either because of iron overload, which alters macrophage physiology and phagocytic properties, or because of the overwhelmed scavenging capacity of macrophages by excessive endometrial reflux (20, 48, 67). In addition, endometrial cells from women with endometriosis have shown molecular and biochemical differences relative to endometrial cells from healthy women (68–70). Consequently, iron homeostasis in endometrial cells from women with endometriosis and in endometriotic cells could differ from that in endometrial healthy cells, and this topic is being researched in our laboratory as a possible mechanism to explain why endometriosis occurs only in some but not in all women with retrograde menstruation. For instance, altered expression of transporters that mediate cellular entry and exit of iron could favor increased iron concentration in endometrial cells, possibly triggering the responses observed in the present study. These postulates may explain in part the origin and progression of the disease, but many other known factors have been shown to be important contributing etiologic mechanisms of endometriosis (65, 71–73).

The iron overload–modulated NF-κB activation, ICAM-1 expression, and sICAM-1 secretion by ESCs shown in this study suggest them as a mechanism inducing and/or promoting endometriosis-associated inflammation and endometriosis induction and development. Together these results suggest that the iron overload observed in the peritoneal cavity of patients with endometriosis (21–24) may trigger and maintain the NF-κB constitutive activation shown in peritoneal endometriotic lesions (44) as well as activate NF-κB in refluxed endometrial cells during menstruation, increasing the inflammatory response by ectopic endometrial cells. Other known NF-κB-mediated cell responses, such as promotion of cell proliferation, inhibition of apoptosis, angiogenesis, and tissue invasion (2, 4, 11, 12, 74, 75), could be mediated by iron overload in endometrial cells and contribute to their proendometriotic phenotype transformation, but this remains to be studied in future investigations. Likewise, even if these experiments show

NF-κB activation and ICAM-1 overexpression in response to iron overload in ESCs, since we did not assess ICAM-1 expression in response to NF-κB inhibition, the possibility of other pathways modulating an iron-dependent ICAM-1 increase cannot be excluded and should be addressed in the future. Another limitation of this work is that, as in many in vitro studies, the conditions of the intraperitoneal environment in vivo cannot be equaled and undoubtedly are much more complex in the presence of hundreds of other cell mediators, such as hormones and cytokines, among others.

In summary, the results of this study propose a role of iron overload as a proinflammatory NF-κB pathway activator in ESCs, conferring proendometriotic behavior on ESCs. This cell signaling mechanism may account for the initiating or evolving processes of this chronic disease. In this context, the search for new strategies focused on diminishing the local influence of iron overload could help in the prevention and treatment of endometriosis.

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