

# Tumor Necrosis Factor- $\alpha$ -Induced Cyclooxygenase-2 Overexpression in Eutopic Endometrium of Women with Endometriosis by Stromal Cell Culture Through Nuclear Factor- $\kappa$ B Activation

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**OBJECTIVE:** To investigate the role of NF- $\kappa$ B during the induction of COX-2 by TNF- $\alpha$  in the eutopic endometrium of women with and without endometriosis using stromal cell culture.

**STUDY DESIGN:** Experimental study in cultured stromal cells of the eutopic endometrium of women with and without endometriosis in the presence of TNF- $\alpha$ .

**RESULTS:** Within 5–20 minutes of stimulation with TNF- $\alpha$ , p-I $\kappa$ B was clearly observed in the eutopic endometrium of women with endometriosis and in that of women without endometriosis. COX-2 protein was significantly induced by treatment with TNF- $\alpha$  in both eutopic endometrial stromal cells of women with and without endometriosis ( $p < 0.05$ ), but the degree of induction was significantly increased in eutopic endometrial stromal cells of women with endometriosis than those of

women without endometriosis ( $p < 0.05$ ).

**CONCLUSION:** Overexpression of COX-2 by TNF- $\alpha$  in the eutopic endometrium of women with endometriosis may play a critical role in such pathophysiologic processes as endometriosis formation. (J Reprod Med 2009;54:625–630)

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**Increased sensitivity of TNF- $\alpha$ -dependent COX-2 expression in eutopic endometrial stromal cells from women with endometriosis may play a critical role in the pathophysiology of endometriosis development.**

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**Keywords:** cyclooxygenase-2, endometriosis, endometrium, NF- $\kappa$ B, stromal cell culture, TNF- $\alpha$ .

Endometriosis is considered to be an inflammationlike phenomenon.<sup>1</sup> Inflammatory responses are now thought to be mediated by the activation of the transcription factor, nuclear factor- $\kappa$ B (NF- $\kappa$ B). NF- $\kappa$ B can be activated by different stimuli, including proinflammatory cytokines. Tumor necrosis factor (TNF)- $\alpha$  is known as a pluripotent mediator that

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promotes the production of other cytokines in various cells. NF- $\kappa$ B activation enhances the transcription of TNF- $\alpha$ , and TNF- $\alpha$ , in turn, is known to activate NF- $\kappa$ B.<sup>2</sup>

A recent study demonstrated that the eutopic endometrium and ectopic endometriotic implants in women with endometriosis express more cyclooxygenase-2 (COX-2) than the endometrium of disease-free women,<sup>3,4</sup> indicating that overexpression of COX-2 may be associated with this disorder. However, the mechanism responsible for elevation of COX-2 in ectopic endometriotic implants remains uncharacterized.

Therefore, we hypothesize that TNF- $\alpha$  may induce COX-2 production in the eutopic endometrium of women with endometriosis through NF- $\kappa$ B activation.

In this study we investigated the role of NF- $\kappa$ B during the induction of COX-2 by TNF- $\alpha$  in the eutopic endometrium of women with endometriosis and that of women without endometriosis, using stromal cell culture. In addition, we compared the effects of TNF- $\alpha$  on the expression of COX-2 between the eutopic endometrium of women with endometriosis and the eutopic endometrium from endometriosis-free women treated with TNF- $\alpha$  by stromal cell culture.

## Materials and Methods

### Clinical Subjects and Samples

With patients' informed consent, human endometrial tissues were obtained by curettage of hysterectomy specimens from patients with myoma (n = 10) and endometriosis (n = 8) who were estimated to be in the mid or late proliferative phase of the menstrual cycle. All samples were histologically confirmed. Among the 18 patients who did not receive preoperative gonadotropin-releasing hormone (GnRH) agonist treatment, 3 women had stage III disease, and 5 had stage IV disease. The clinical

characteristics of these patients are presented in Table I.

### Reagents and Antibodies

The primary and secondary antiphosphorylated inhibitor  $\kappa$ B (anti-p-I $\kappa$ B) antibodies used in this study were the I $\kappa$ B- $\alpha$  (Ser<sup>32</sup>) antibody kit (Cell Signaling Technology, Inc., Beverly, Massachusetts). The antibody against COX-2 was purchased from Santa Cruz Biotechnology (Santa Cruz, California). The antibody against  $\beta$ -actin was purchased from Sigma (St. Louis, Missouri). The antibodies against goat horseradish peroxidase (HRP) and mouse HRP were purchased from Chemicon (Temecula, California).

### Cell Cultures and Treatment

Stromal cells were cultured using a modified protocol previously reported by Osteene et al<sup>5</sup> with minor modifications. Then the tissues were minced in 2–3 mL of Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, Grand Island, New York). The tissues were centrifuged at 85 g in a conical tube, 10 mL of trypsin-EDTA (Gibco) was added to the pellet, and the pellet was incubated in a shaker at 37°C. After 30 minutes of incubation, 1 mL of 10% heat-inactivated fetal bovine serum (FBS) (Gibco) was added to stop the enzyme reaction. The tissues were centrifuged and the upper portion removed. The tissue was once more suspended and washed with a phosphate-buffered saline, and then the cells were resuspended in 10 mL DMEM/F-12 with 10% FBS. After 20 minutes at room temperature, the upper two thirds of the supernatant, enriched in stromal cells, were filtered through a 40-mm nylon mesh (BD Falcon, San Jose, California) filter for final purification from any remaining clumps of epithelial cells. The filtered, stromal-enriched fraction was seeded on a 60-mm culture dish (Corning, New York) for 24 hours. After 24 hours, the medium was changed to remove unattached cells, and the cultures were allowed to proliferate until confluence (24–72 hours), with the medium exchanged every 24 hours.

To determine the purity of the stromal cell preparation, it was characterized using immunostaining with antibodies to cytokeratin (Santa Cruz) and vimentin (Santa Cruz). The purity of the stromal cells was >98%.

The cells were cultured for 24 hours in serum-free DMEM/F12 medium (Gibco), and then TNF- $\alpha$  (0.1 ng/m, Genzyme Techno, Minneapolis, Minnesota)

**Table I** Clinical Characteristics in this Study

Characteristic	Myoma (n = 10)	Endometriosis (n = 8)	p Value
Age (yr)	41.11 $\pm$ 4.53	42.12 $\pm$ 4.70	>0.05
Gravida	1.93 $\pm$ 1.44	3.63 $\pm$ 4.92	0.007
Parity	1.29 $\pm$ 0.99	1.79 $\pm$ 0.54	>0.05
BMI	22.64 $\pm$ 2.67	24.17 $\pm$ 3.84	>0.05

All results are mean  $\pm$  SD. BMI = body mass index.

was added to the medium for the indicated times in time course experiments.

#### Western Blot Analysis of Phosphorylated-I $\kappa$ B and COX-2

Western blot analysis was performed as described. Briefly, cell lysates were prepared by extracting proteins with lysis buffer (40 mM Tris-Cl, pH 8.0; 120 mM NaCl; 0.1% Nonidet-P40 ) supplemented with protease inhibitors. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Satorius AG, Goettingen, Germany). Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence procedures (Santa Cruz) according to the manufacturer's recommendations. The Western blot analyses were repeated at least 3 times, and representative data are shown. The expression level of COX-2 was determined by Western blot analysis using the densitometric value (SCION image program, Washington, D.C.). The values of COX-2 levels, using the  $\beta$ -actin expression level for standardization, are shown as ratios.

#### Collection of Supernatants After Culture with TNF- $\alpha$

To measure the secretion of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) from cells in different conditions after 24 hours in culture, the cells were removed by centrifugation, and the cell-free media were collected and stored at -75°C until use. After the media were thawed at room temperature, they were assayed for immunoreactive PGE<sub>2</sub> by enzyme-linked immunosorbent assay (ELISA) with anti-PGE<sub>2</sub> antibodies (ELISA kit, Sigma) according to the manufacturer's specifications with absorbance at 450 nm in a plate reader (SpectraMax 190, Molecular Device, Sunnyvale, California).

#### Effects of NF- $\kappa$ B Inhibitor on the Expression of COX-2 mRNA

Eutopic endometrial stromal cells were plated in a 60-mm culture dish as described above. After the cells were preincubated in phenol red-free medium without serum for 24 hours at 37°C, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (10<sup>-5</sup> M) (Sigma-Aldrich), an NF- $\kappa$ B inhibitor, was added and incubated for 1 hour. Then TNF- $\alpha$  (0.1 ng/mL) was added and incubated for 24 hours. Total RNA was extracted from cultured stromal cells using an Ultraspec RNA isolation kit (Biotec, Houston, Texas) according to the manufacturer's instruc-

tions. RNA concentration was determined spectrophotometrically. Human COX-2 primers were 5'-CCACCAAC-TTACAATGCTGC-3' (sense) and 5'-CACCAGACCAAAGACCTAA-3' (antisense). The resultant polymerase chain reaction products were resolved on a 1.5% agarose gel. After staining with ethidium bromide, the gel was photographed, and the intensity of the signal was analyzed using the SCION image program. The values for COX-2 levels, using the GAPDH expression level for standardization, are shown as ratios.

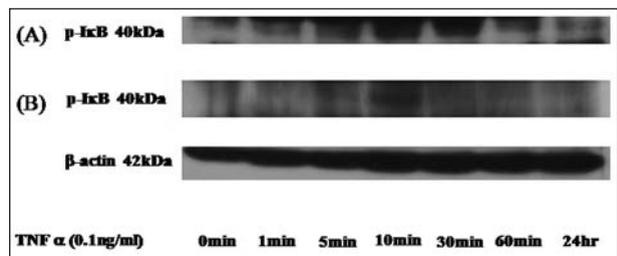
#### Statistical Analysis

Where appropriate, the results are expressed as mean  $\pm$  SD and were statistically analyzed using an unpaired *t* test and ANOVA (1-way analysis of variance) followed by Tukey's test using SAS 9.1.3 statistical software (SAS Institute, Inc., Cary, North Carolina). A probability level of  $p < 0.05$  was considered significant.

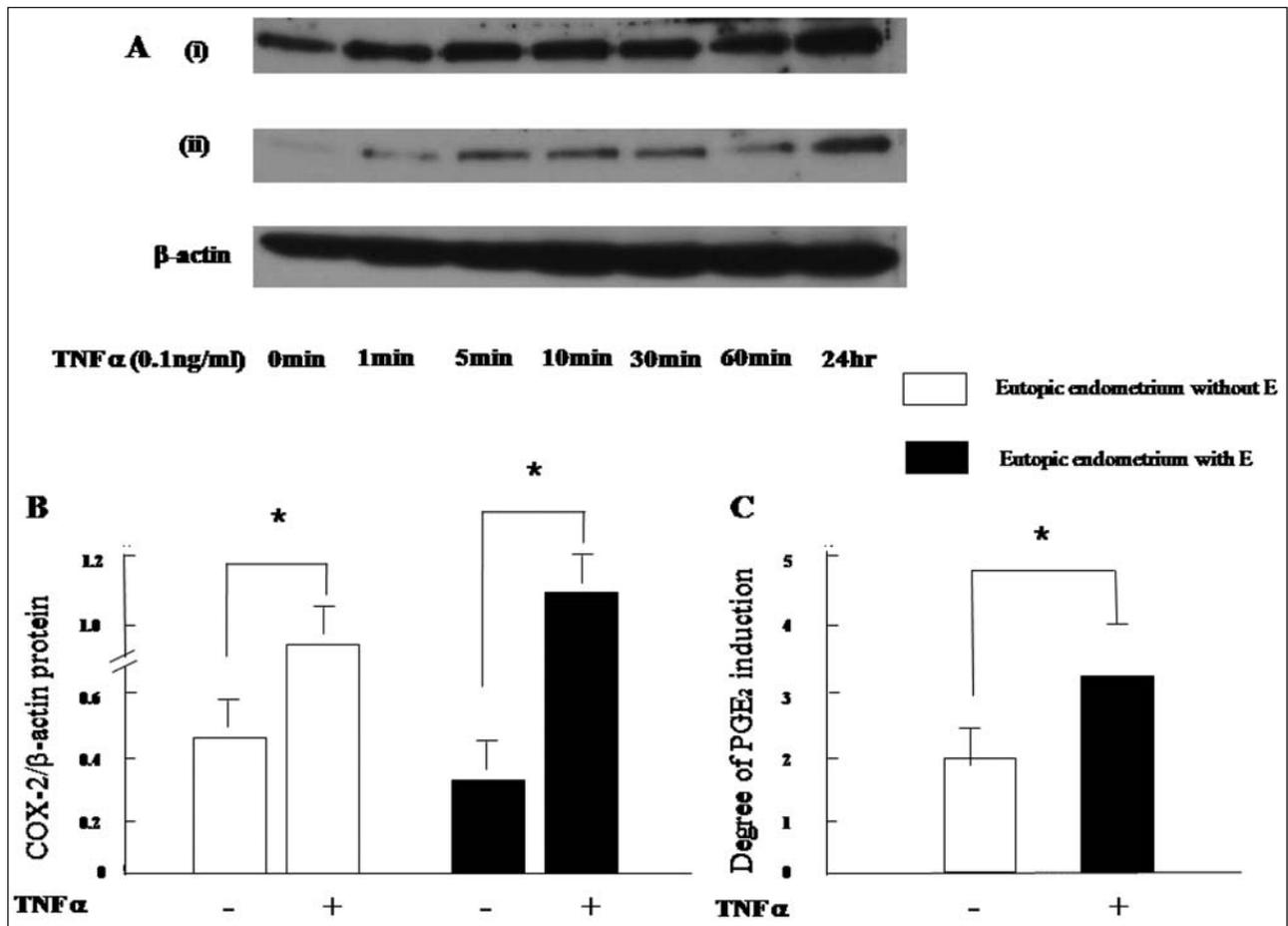
#### Results

Within 5–20 minutes of stimulation with TNF- $\alpha$ , p-I $\kappa$ B was clearly observed in the eutopic endometrium of patients with endometriosis and of patients without endometriosis (Figure 1).

In the absence of TNF- $\alpha$ , COX-2 protein was detected in the eutopic endometrial stromal cells in patients with endometriosis and without endometriosis (Figure 2A). The basal level of COX-2 in stromal cells derived from endometriosis-free endometrium was greater than that in stromal cells from the endometrium of patients with endometriosis ( $0.45 \pm 0.03$  vs.  $0.33 \pm 0.03$ ,  $p < 0.05$ ). Notably, COX-2 protein was significantly induced by treatment with TNF- $\alpha$  in both eutopic endometrial stromal cells with ( $0.33 \pm 0.03$  vs.  $1.02 \pm 0.05$ ,  $p < 0.05$ ) and without



**Figure 1** Time-course analysis of TNF- $\alpha$ -induced phosphorylation of I $\kappa$ B in eutopic endometrial stromal cells of women (A) without endometriosis and women (B) with endometriosis.



**Figure 2** Effects of TNF- $\alpha$  on COX-2 protein production in eutopic endometrial stromal cells of women (i) without endometriosis and (ii) with endometriosis. Stromal cells were exposed to either medium alone or medium with TNF- $\alpha$  (0.1 ng/mL) for 24 hours. (A) A representative Western blot showing that the expression of COX-2 is markedly increased in eutopic endometrial stromal cells of women (ii) with endometriosis over that in cells of women (i) without endometriosis. (B) COX-2 protein levels were determined by Western blot analysis using the densitometric value. Data are means  $\pm$  SD, and asterisks denote significant difference. (C) Effects of TNF- $\alpha$  on PGE<sub>2</sub> production in eutopic endometrial stromal cells of women (i) without endometriosis and women (ii) with endometriosis. Stromal cells were exposed to either medium alone or medium with TNF- $\alpha$  (0.1 ng/mL) for 24 hours. Concentrations of PGE<sub>2</sub> in the supernatants were measured by enzyme-linked immunosorbent assay. The bar represents the degree of PGE<sub>2</sub> induction. Asterisks denote a significant difference between eutopic endometrial stromal cells of women with and without endometriosis.

endometriosis ( $0.45 \pm 0.30$  vs.  $0.90 \pm 0.01$ ,  $p < 0.05$ ), but the degree of induction was significantly increased in eutopic endometrial stromal cells from women with endometriosis than those from women without endometriosis ( $3.09 \pm 0.25$  vs.  $1.99 \pm 0.12$ ,  $p < 0.05$ ) (Figure 2B).

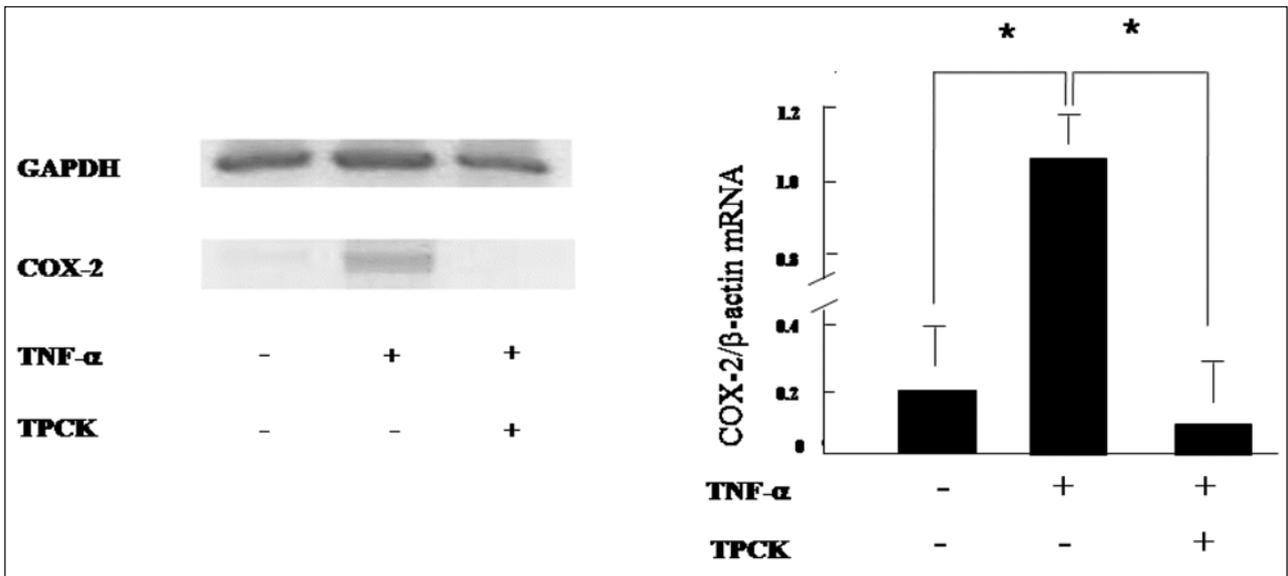
The degree of PGE<sub>2</sub> induction was significantly increased in eutopic endometrial stromal cells from women with than those from women without endometriosis ( $3.90 \pm 0.80$  vs.  $1.79 \pm 0.33$ ,  $p < 0.05$ ) (Figure 2C).

Pretreatment with TPCK, an NF- $\kappa$ B inhibitor, re-

duced TNF- $\alpha$ -induced COX-2 mRNA expression ( $1.18 \pm 0.00$  vs.  $0.09 \pm 0.00$ ,  $p < 0.05$ ) (Figure 3).

### Discussion

Yoshino et al<sup>6</sup> reported that IL-1 $\beta$ -induced COX-2 expression was suppressed by inhibitors of ERK and p38. The report suggested that MAPKs may play possible pathophysiologic roles in endometriosis, acting as intracellular signal transducers. Sakamoto et al<sup>7</sup> reported that NF- $\kappa$ B activation is critical for TNF- $\alpha$ -induced IL-8 expression in endometriotic stromal cells. Wu et al<sup>8</sup> further showed



**Figure 3** Effect of the NF- $\kappa$ B inhibitor N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) on TNF- $\alpha$ -induced COX-2 mRNA expression. Confluent eutopic endometrial stromal cells were preincubated in medium without serum for 24 hours at 37°C, and TPCK 10<sup>-5</sup> M (Sigma-Aldrich) was added and incubated for 1 hour. Then TNF- $\alpha$  (0.1 ng/mL) (Chemicon) was added and incubated for 24 hours. Total RNA was isolated from the cells and analyzed by reverse transcriptase polymerase chain reaction. Asterisks denote significant difference between groups.

that the endometrial stromal cells undergo phosphorylation under the stimulation of TNF- $\alpha$  and that specific inhibitors of NF- $\kappa$ B inhibit TNF- $\alpha$ -induced COX-2 protein expression.

A continuing debate on the pathophysiology of the disease concerns whether endometriosis implants are simply translocations of normal endometrium to ectopic sites or whether the cells that comprise these implants are significantly different from their counterparts. Tseng et al<sup>9</sup> demonstrated that eutopic endometrial stromal cells of women with endometriosis secrete more IL-6 than cells from disease-free, normal, control women, suggesting that endometrial stromal cells of women with endometriosis differ intrinsically in terms of their ability to produce IL-6. Therefore, intrinsic molecular aberrations and increased sensitivity to proinflammatory cytokines of eutopic endometrium of women with endometriosis are considered to contribute significantly to the development of endometriosis.

However, whether TNF- $\alpha$  differentially affects COX-2 expression in endometrial stromal cells of women with endometriosis and without endometriosis remains unclear.

Wu et al<sup>8</sup> reported that induction of COX-2 by

IL-1 $\beta$  in ectopic endometriotic stromal cells is at least 100 times more sensitive than compared with eutopic endometrium stromal cells. The researchers explained that the induction of COX-2 expression by IL-1 $\beta$  is through transcriptional regulation of the COX-2 gene via activation of the ERK/p38 MAPK-dependent cAMP response element binding site phosphorylation in ectopic endometrial stromal cells and posttranscriptional enhancement of COX-2 mRNA stability.

We showed that TNF- $\alpha$  increased COX-2 expression and concomitant PGE<sub>2</sub> production in eutopic endometrium of women with and without endometriosis. The induction of COX-2 expression by TNF- $\alpha$  is through regulation of COX-2 protein via activation of NF- $\kappa$ B in eutopic endometrial stromal cells. Increased sensitivity of TNF- $\alpha$ -dependent COX-2 expression in eutopic endometrial stromal cells from women with endometriosis may play a critical role in the pathophysiology of endometriosis development.

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