# In vitro effects of atorvastatin on lipopolysaccharide-induced gene expression in endometriotic stromal cells

*Indu Sharma, M.Sc.,*[a] *Veena Dhawan, Ph.D.,*[a] *Nitin Mahajan, Ph.D.,*[a] *Subhash Chand Saha, M.D.,*[b] *and Lakhbir Kaur Dhaliwal, M.D.*[b]

[a] Department of Experimental Medicine and Biotechnology; and [b] Department of Obstetrics and Gynecology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

**Objective:** To investigate the in vitro effects of atorvastatin on lipopolysaccharide (LPS)-induced gene expression in endometrial-endometriotic stromal cells.
**Design:** In vitro experimental study using flow cytometry, ELISA, semiquantitative reverse transcriptase polymerase chain reaction, and Western blot.
**Setting:** Postgraduate Institute of Medical Education and Research.
**Patient(s):** Twenty-five women undergoing laparoscopy (n = 10) and laparotomy (n = 15).
**Intervention(s):** Endometriotic cyst wall (group I) and endometrial biopsy (group II) collection.
**Main Outcome Measure(s):** The endometrial-endometriotic stromal cells were isolated from ectopic (group I) and eutopic (group II) endometrium by established methods, cultured, and stimulated with LPS (1 $\mu$g/mL), followed by atorvastatin treatment in a time- and dose-dependent manner to investigate the effects of LPS on proliferation (Ki-67) and expression of cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), receptor for advanced glycation end products (RAGE), extracellular newly identified RAGE binding protein (EN-RAGE), peroxisome proliferator activated receptor-$\gamma$ (PPAR-$\gamma$), and liver X receptor-$\alpha$ (LXR-$\alpha$) genes in endometrial-endometriotic stromal cells and on levels of insulin-like growth factor binding protein-1 (IGFBP-1) and 17$\beta$-$E_2$ in endometrial-endometriotic stromal cell culture supernatant.
**Result(s):** Significant inhibition of Ki-67 and LPS-induced expression of inflammatory and angiogenic genes (COX-2, VEGF, RAGE, and EN-RAGE) was observed in atorvastatin-treated endometrial-endometriotic stromal cells. In contrast, a significant dose- and time-dependent increase in expression of anti-inflammatory genes (PPAR-$\gamma$ and LXR-$\alpha$) and levels of IGFBP-1 was observed after atorvastatin treatment in both the groups. However, atorvastatin treatment had no effect on 17$\beta$-$E_2$ levels in endometrial/endometriotic stromal cell culture supernatant.
**Conclusion(s):** The data of the present study provide new insights for the implication of atorvastatin treatment for endometriosis in humans. (Fertil Steril® 2010;94:1639–46. ©2010 by American Society for Reproductive Medicine.)

**Key Words:** Endometriosis, inflammation, ESCs, atorvastatin, in vitro

Endometriosis is defined as the presence of tissue resembling endometrium in extrauterine sites, most commonly the ovaries and the peritoneum. Inflammation and growth are the two pathologic processes that remain associated via a positive feedback cycle and have been shown to be responsible for chronic pelvic pain and infertility in patients with endometriosis (1).

Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme in prostanoid biosynthesis; is involved in inflammation, angiogenesis, cell growth, and differentiation; and is likely to be influential in several of the processes that lead to ectopic endometrial development (2, 3). Central to the survival and growth of endometriotic implants is the establishment of an effective blood supply, a process termed angiogenesis, which is provided and maintained by the proinflammatory milieu of the peritoneal cavity and associated immune cells (4–6).

Tanaka et al. (7) demonstrated that advanced glycation end products, tumor necrosis factor-$\alpha$, and $E_2$ activate expression of receptor for advanced glycation end products (RAGE) through nuclear factor-$\kappa$B and Sp-1, causing advanced glycation end products–RAGE interactions. Because high estrogen levels have been postulated to promote endometriosis (8), there is a high probability that in this milieu RAGE also may be expressed at high levels, which possibly could be a reason for augmented inflammatory response in endometriosis. Among various ligands for this receptor, S100A12, also termed extracellular newly identified RAGE binding protein (EN-RAGE), has a distinct role in inflammation (9, 10). Further, peroxisome proliferator activated receptor-$\gamma$ (PPAR-$\gamma$) and liver X receptor-$\alpha$ (LXR-$\alpha$) have a crucial role in lipid metabolism, cellular differentiation, glucose homeostasis, eicosanoid signaling, and inflammation (11–13). Peroxisome proliferator activated receptor-$\gamma$

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Reprint requests: Veena Dhawan, Ph.D., Associate Professor, Department of Experimental Medicine and Biotechnology, Research Block "B" Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh 160012, India (FAX: 91-172-2744401; E-mail: veenad2001@yahoo.com).

agonists, both natural and synthetic, are known to inhibit tumor growth and metastasis by inhibiting COX-2 and vascular endothelial growth factor (VEGF) (14, 15). Although PPAR-$\gamma$ expression is reported in peritoneal macrophages and endometriotic stromal cells (16, 17), there are no reports regarding the role of LXR-$\alpha$ in endometriosis.

All this evidence conjectures toward an inflammatory etiology of endometriosis. Many in vitro and in vivo studies have demonstrated that statins, a class of drugs, exhibit anti-inflammatory activities and pleiotropic effects that are independent of their cholesterol-lowering properties. Recently, statins also have been shown to inhibit the proliferation and angiogenesis of human endometrial stromal cells in vitro (18, 19). Atorvastatin and other statins also have been shown to activate PPAR-$\gamma$ and LXR-$\alpha$ in primary human monocytes in culture and inhibit the production of inflammatory cytokines (20–23).

We hypothesized that atorvastatin might influence endometriosis by enhancing the expression of anti-inflammatory genes such as PPAR-$\gamma$ and LXR-$\alpha$, which may further elicit their downstream anti-inflammatory, antioxidative, and antiangiogenic effects and obliterate any aberrant behavior of the above-mentioned key molecules in endometriosis. Therefore, in the present study, we investigated the impact of atorvastatin on proliferation, differentiation, 17$\beta$-$E_2$ production, and gene expression in endometriotic stromal cells after lipopolysaccharide (LPS) stimulation.

## MATERIALS AND METHODS

Fifteen women with laparoscopically proved endometriosis and 10 women without endometriosis were included in group 1 and 2 respectively by using the criteria as described previously (24). At the time of laparoscopy (n = 2) or laparotomy (n = 13), endometriotic cyst wall tissue was obtained by biopsy from endometrioma for group 1. For control subjects (group 2), endometrial tissue was obtained in the secretory phase of their menstrual cycle by endometrial biopsy curette performed as a routine protocol in the Infertility Clinic (n = 8) and during hysterectomy (n = 2). Endometriosis was diagnosed with use of the revised classification of the American Fertility Society (25). All the female patients included in this study were of reproductive age (18–40 years), menstruating normally, and normotensive with normal body mass index. None of the subjects was receiving any drug treatment except for one woman in group 1 who had received danazol previously. A fully informed written consent was taken from all the study subjects before their participation in the study. The study was approved by the Institutional Ethics Committee. The tissue sample was taken in the media immediately and was processed for isolation of endometriotic stromal cells and establishment of their primary cell culture.

### Primary Cell Culture

The tissue sample was washed properly with red blood cell lysis buffer, minced, and incubated in fresh media (Dulbecco's minimum essential medium and Ham's F-12; 1:1; vol/vol), collagenase, and deoxyribonuclease (catalog no. RM2075 and catalog no. RM2090; HiMedia Laboratories Pvt. Ltd., Mumbai, India) for 2 hours at 37°C with constant agitation. The dispersed endometriotic stromal cells were separated by serial filtration through nylon net filters of pore size 80 $\mu$m and 40 $\mu$m (catalog no. NY80 025 00 and catalog no. NY40 025 00; Millipore Corporation, Milford, MA) and plated at a density of approximately $10^6$ cells per milliliter of media. Morphologically, the isolated endometriotic stromal cells were elongated and fibroblast-like in appearance (Fig. 1A). The endometriotic stromal cells were maintained at 37°C in a humidified atmosphere with 5% $CO_2$ and were allowed to confluence for approximately 72 hours. Thereafter, endometriotic stromal cells were passaged at least three times by trypsinization and plated in 24-well plates at a density of approximately $2 \times 10^5$ cells per milliliter of media with charcoal-treated fetal bovine serum before the start of in vitro treatment (LPS-atorvastatin). The presence of endometriotic stromal cells was confirmed by fluorescence-activated cell sorter with use of fluorescein isothiocyanate conjugate–labeled antivimentin (catalog no. sc-32322; Santa Cruz Biotechnology, Santa Cruz, CA) and phycoerythrin-labeled anti-CD10 (catalog no. 555375; BD Biosciences, San Jose, CA) antibodies. The cultured endometriotic stromal cells were found to be >95% positive for both antibodies (Fig. 1B). These cells also were found to be positive for expression of estrogen receptor (ER)-$\alpha$ at the protein level by Western blot analysis (Fig. 1F).

### In Vitro Treatment of Endometriotic Stromal Cells

The cultured endometriotic stromal cells were stimulated with LPS (1 $\mu$g/mL) and then incubated with different doses of atorvastatin (0–40 $\mu$mol/L/$2 \times 10^5$ cells per milliliter of media) for different time periods (0, 6, 9, 12, and 24 hours). Atorvastatin was purchased commercially from Cipla Pvt. Ltd, Mumbai, India. After incubation, the cells were harvested and centrifuged for 10 minutes at 2000 rpm. The supernatants were saved and stored at −20°C for further analysis. The cell pellet was processed to determine the expression of test genes (COX-2, VEGF, RAGE, EN-RAGE, PPAR-$\gamma$, LXR-$\alpha$) at both the messenger RNA (mRNA) and protein level.

### Cell Viability and Cell Proliferation

Cell viability was assessed by trypan blue exclusion assay (26), and cell proliferation was assessed by fluorescence-activated cell sorter using fluorescein isothiocyanate conjugate–labeled anti-Ki-67 antibody (catalog No. 556026; BD Biosciences).

### Cell Differentiation

Insulin-like growth factor binding protein-1 (IGFBP-1), a marker for endometrial stromal cell differentiation, was analyzed in the culture supernatants with use of a commercially available ELISA kit (catalog no. ELH-IGFBP1-001; RayBiotech, Norcross, GA). The minimum detectable concentration of IGFBP-1 was 2.74 pg/mL; therefore, values observed below this concentration were considered as undetectable.

### Determination of 17$\beta$-$E_2$ levels

Levels of 17$\beta$-$E_2$ were determined in the culture supernatants with use of a commercially available enzyme immunoassay kit (catalog no. DKO 003; Diametra, Foligno, Italy). The minimum detectable concentration of the assay was 20 pg/mL; thus, the values observed below this concentration were considered as undetectable.

### Reverse Transcriptase–Polymerase Chain Reaction Studies

Semiquantitative reverse transcriptase–polymerase chain reaction was performed to determine the mRNA expression of the test genes in atorvastatin-treated endometriotic stromal cells (10, 27) with use of human-specific primer pairs (Supplementary Table 1).

### Western Blot Analysis

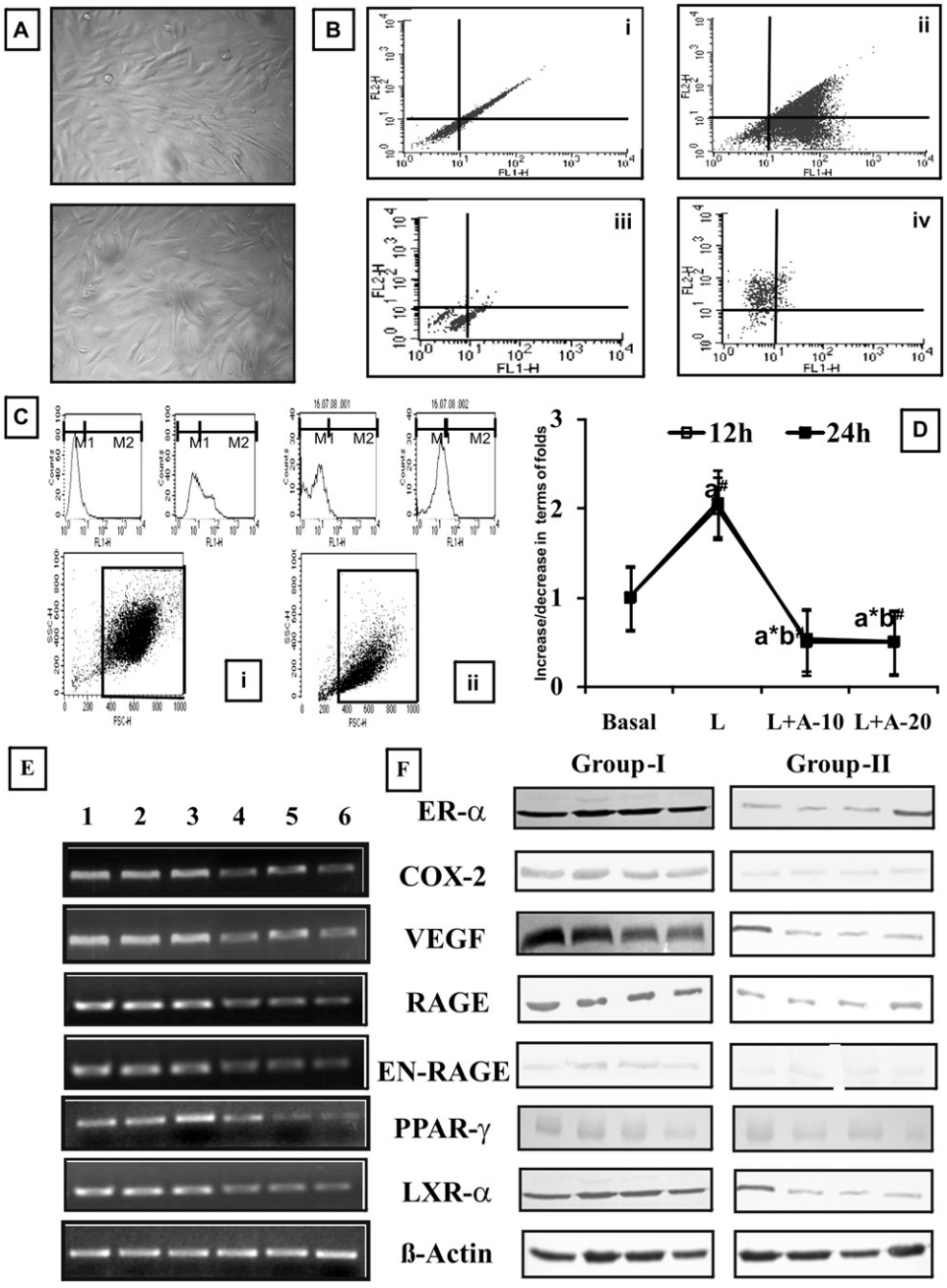
Protein expression of all the test genes was determined by Western blot analysis with use of mouse anti-$\beta$-actin (catalog no. AU1978; Sigma Chemical Co., St. Louis, MO), mouse anti-ER-$\alpha$ (catalog no. sc-8002; Santa Cruz Biotechnology), mouse anti-COX-2 (catalog no. 236004; CalBiochem, Darmstadt, Germany), rabbit anti-VEGF (catalog no. V6627; Sigma Chemical Co.), goat anti-RAGE (catalog no. AF1145; R&D Systems, Minneapolis, MN), goat anti-EN-RAGE (catalog no. AF1052; R&D Systems), goat anti-PPAR-$\gamma$ (catalog no. sc-6285; Santa Cruz Biotechnology), and goat anti-LXR-$\alpha$ (catalog no. sc-1201; Santa Cruz Biotechnology) antibodies separately. The secondary antibodies used were anti-mouse (catalog no. A9044; Sigma Chemical Co., St. Louis, MO), anti-goat (catalog no. A 5420; Sigma Chemical Co.), and anti-rabbit horseradish peroxidase–conjugated (catalog no. A 0545; Sigma Chemical Co.).

### Statistical Analysis

The analyses were performed with use of the statistical software, SPSS 10.0 (SPSS Inc., Chicago, IL). All results are represented as mean ± SEM.

## FIGURE 1

(**A**) Morphology of endometriotic-endometrial stromal cells in vitro. The isolated endometriotic-endometrial stromal cells were elongated and fibroblast-like in appearance. (**B**) Dot plot for fluorescence-activated cell sorter analysis of endometriotic stromal cells. i, iii = Unlabeled; ii = labeled with fluorescein isothiocyanate conjugate–antivimentin antibody; iv = labeled with Phycoerythrin–anti-CD10 antibody. (**C**) Fluorescence-activated cell sorter analysis for Ki-67 as an index of cell proliferation in endometriotic stromal cells of group 1 (i) and 2 (ii) subjects. (**D**) Effect of atorvastatin on cell proliferation of LPS-induced endometriotic stromal cells in vitro. L = LPS (1 $\mu$g/mL); L+A-10 = LPS (1 $\mu$g/mL) + atorvastatin (10 $\mu$mol/L); L+A-20 = LPS (1 $\mu$g/mL) + atorvastatin (20 $\mu$mol/L); 12 h = 12 hours; 24 h = 24 hours; a = comparison versus basal; b = versus L. $^{*}P<.05$; $^{\#}P<.001$. (**E**) Representative agarose gel photographs depicting mRNA expression of test genes (COX-2, VEGF, RAGE, EN-RAGE, PPAR-$\gamma$, LXR-$\alpha$, and $\beta$-actin) in group 1 (lanes 1–3) and 2 (lanes 4–6) study subjects. (**F**) Representative photographs depicting protein expression of test genes (COX-2, VEGF, RAGE, EN-RAGE, PPAR-$\gamma$, LXR-$\alpha$, and $\beta$-actin) in group 1 and 2 study subjects.

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Comparison of variables between the two study groups was carried out with use of unpaired Student's *t*-test and ANOVA as and when appropriate with a significance threshold *P* value of .05.

## RESULTS

### Effect of Atorvastatin on Cell Viability and Proliferation

Atorvastatin in a dose of 20 $\mu$mol/L had no effect on the viability of endometriotic stromal cells up to 24 hours. However, higher doses led to decreased viability at 48 hours and 72 hours (<95%; data not shown). No morphologic abnormalities were found in endometriotic stromal cell cultures with a similar dose. On the basis of these observations and keeping in mind the pharmacokinetics of atorvastatin, for further experiments we used atorvastatin in a maximum dose of 20 $\mu$mol/L, and the experiments were carried out up to 24 hours only.

Our data demonstrated that atorvastatin was able to inhibit LPS-induced cell proliferation of endometriotic stromal cells as evidenced by a significant decrease in Ki-67 labeling index (Fig. 1C). However, there was no significant difference between the doses and time period studied as far as inhibition of cell proliferation is concerned ($P>.05$; Fig. 1D).

### Effects of Atorvastatin on Cell Differentiation

Similar to the observations for cell proliferation, atorvastatin demonstrated its efficacy in inducing cell differentiation of endometriotic stromal cells. Atorvastatin treatment in a dose of 10 and 20 $\mu$M caused a significant increase in IGFBP-1 levels (picograms per milliliter) at 9 hours in endometriotic cell culture supernatant of group 1 subjects ($P<.05$; Table 1). Beyond 9 hours there were no significant differences in IGFBP-1 levels with any dose of the atorvastatin tested till 24 hours ($P>.05$). However, in group 2 endometriotic stromal cells, the IGFBP-1 levels remained undetectable, and, therefore, the effect of atorvastatin could not be assessed.

### Effects of Atorvastatin on Levels of 17$\beta$-$E_2$

Levels of 17$\beta$-$E_2$ were determined in the cell culture supernatant of atorvastatin-treated endometriotic stromal cells of group 1 and 2 subjects till 48 hours. As the levels of 17$\beta$-$E_2$ remained undetectable in the cell culture supernatant at all time intervals, the effect of atorvastatin could not be assessed (Table 1).

### In Vitro Effects of Atorvastatin on LPS-induced Gene Expression

Next, we examined the time- and dose-dependent effects of atorvastatin treatment on LPS-induced in vitro expression of COX-2, VEGF, RAGE, EN-RAGE, PPAR-$\gamma$, and LXR-$\alpha$ genes in endometriotic stromal cells at both mRNA and protein levels (Fig. 1E and F). As depicted in Figs. 2 and 3, atorvastatin treatment significantly attenuated the expression of both inflammatory and angiogenic genes in both a time- and dose-dependent manner ($P<.05$). On the contrary, atorvastatin treatment caused a significant increase in the mRNA and protein expression of anti-inflammatory genes PPAR-$\gamma$ and LXR-$\alpha$, and the effect of the drug was both dose- and time-dependent ($P<.05$; Figs. 2 and 3).

A maximum and statistically significant decrease in the expression of inflammatory genes in LPS-induced endometriotic stromal cells of patients was observed with a dose of 20 $\mu$mol/L of atorvastatin at 24 hours at both mRNA and protein levels ($P<.05$; Figs. 2 and 3). However, no significant difference was observed between 10 $\mu$mol/L and 20 $\mu$mol/L doses of atorvastatin at 12 and 24 hours. As far as group 2 is concerned, even a minimum dose (5 $\mu$mol/L) of the drug was able to significantly attenuate the LPS-induced mRNA and protein expression of COX-2, VEGF, and EN-RAGE genes, whereas a 10 $\mu$mol/L dose was sufficient in the case of RAGE gene ($P<.05$). A maximum increase in mRNA and protein expression of PPAR-$\gamma$ and LXR-$\alpha$ genes was observed at 9 hours with a 20 $\mu$mol/L dose of atorvastatin ($P<.001$; Figs. 2 and 3). Also, the increase in PPAR-$\gamma$ and LXR-$\alpha$ expression after atorvastatin treatment was more pronounced in endometriotic stromal cells from group 2 subjects at both mRNA and protein levels.

Atorvastatin was dissolved in 0.05% dimethyl sulfoxide, and no significant effect of dimethyl sulfoxide was observed on the expression of target genes in vitro at any time interval, when used as a control (data not shown).

## DISCUSSION

Endometriosis is an ambiguous disease and entails inflammation in its pathophysiology. To date, there is no known cure, and most current medical treatments deal with the limit of preventing spontaneous ovulation and of recurrence after discontinuation and are not suitable for the long term because of their side effects (28). Therefore, development of nonhormonal medical treatments to prevent or treat endometriosis and associated symptoms is a priority (29). In this context, our study highlights the therapeutic role of atorvastatin as an anti-inflammatory and antiangiogenic drug in conditions such as endometriosis.

Although endometriotic lesions are benign, they share certain characteristics with malignancies. Cells of the endometriotic implants have an inherent higher capacity to proliferate, as evidenced by increased Ki-67 index of group 1 endometriotic stromal cells in our study. Further, atorvastatin at a dose of 10 $\mu$mol/L for 12 hours significantly inhibited Ki-67 expression in LPS-induced endometriotic stromal cells of group 1. Our findings are supported by the report of Piotrowski et al. (18), who demonstrated that mevastatin and simvastatin caused a concentration-dependent inhibition of DNA synthesis in human endometriotic stromal cells possibly via inhibition of MAPK3/1 phosphorylation. Statins are known to inhibit Ras isoprenylation via their non–lipid-mediated effects (30, 31). Likely, a similar mechanism may exist in our study as atorvastatin reduced the proliferation of endometriotic stromal cells.

Insulin-like growth factor binding protein-1 levels were low in the culture supernatant of group 1 endometriotic stromal cells as compared with the control group. Insulin-like growth factor binding protein-1 frequently is used as a marker for cell differentiation and plays important roles in ovarian, endometrial, trophoblast, and fetoplacental physiology and pathology (32–34). Thus, finding lower IGFBP-1 levels in the patient group suggests reduced capacity of endometriotic stromal cells for differentiation at ectopic sites and an increased capacity to proliferate, which is also evidenced by a higher Ki-67 index of the same cells. Our observations are in agreement with the findings of Klemmt et al. (35), who demonstrated lower levels of IGFBP-1 in endometriotic stromal cells from subjects with endometriosis, and Kim et al. (36), who observed higher insulin-like growth factor-1 levels in similar subjects.

Atorvastatin treatment significantly induced differentiation of endometriotic stromal cells in vitro as observed by finding increased levels of IGFBP-1 in the cell culture supernatant. This could be speculated as favorable in endometriotic disease, as increased cell differentiation would interfere with growth and replenishment of endometriotic stromal cells at ectopic sites. However, to date, there is no report available in the literature that demonstrates the effect of atorvastatin on IGFBP-1 levels.

**TABLE 1**

**Levels of IGFBP-1 and 17$\beta$-$E_2$ in the cell culture supernatant of LPS-induced endometriotic stromal cells after atorvastatin treatment.**

| Atorvastatin treatment | IGFBP-1 (pg/mL) | | 17$\beta$-$E_2$ (pg/mL) | |
|---|---|---|---|---|
| | Group 1 | Group 2 | Group 1 | Group 2 |
| Basal | 5 | 11 | — | — |
| 6 h | | | | |
| L | UD | UD | — | — |
| L+A-10 | UD | UD | — | — |
| L+A-20 | UD | UD | — | — |
| 9 h | | | | |
| L | 5.2 | UD | — | — |
| L+A-10 | 5 | UD | — | — |
| L+A-20 | 9.9[a] | UD | — | — |
| 12 h | | | | |
| L | 6 | UD | UD | UD |
| L+A-10 | 9.5[a] | UD | UD | UD |
| L+A-20 | 9.8[a] | UD | UD | UD |
| 24 h | | | | |
| L | 8 | UD | UD | UD |
| L+A-10 | 9.6[a] | UD | UD | UD |
| L+A-20 | 9.9[a] | UD | UD | UD |
| 48 h | | | | |
| L | — | — | UD | UD |
| L+A-10 | — | — | 22 | UD |
| L+A-20 | — | — | UD | UD |

*Note:* Data presented are mean of three similar experiments. L = LPS (1 $\mu$g/mL); L+A-10 = LPS + atorvastatin (10 $\mu$mol/L); L+A-20 = LPS + atorvastatin (20 $\mu$mol/L); UD = undetectable.

[a] $P<.05$, compared with group 2.

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As hypothesized, atorvastatin treatment of endometriotic stromal cells proved to be of potential benefit because it significantly inhibited the expression of genes with known inflammatory potential. A maximum and significant decrease in LPS-induced gene expression at both mRNA and protein levels was observed at 24 hours with a higher dose of atorvastatin in endometriotic stromal cells from the patient group, whereas, for a similar effect, a comparatively lesser dose of the drug was required in group 2 subjects. These observations suggested that inflammation was prevalent in the patient group. Our findings are in accordance with a few reports in other pathologic disorders where authors reported down-regulation of COX-2 (37–39), VEGF (40, 41), RAGE (27, 42), and EN-RAGE (27) genes with atorvastatin and other statins in both in vitro and in vivo conditions. Also, ours is the first report that highlights the fact that atorvastatin significantly attenuates expression of inflammatory genes in endometriosis and reemphasizes important anti-inflammatory, antiangiogenic, and antioxidative properties of atorvastatin.

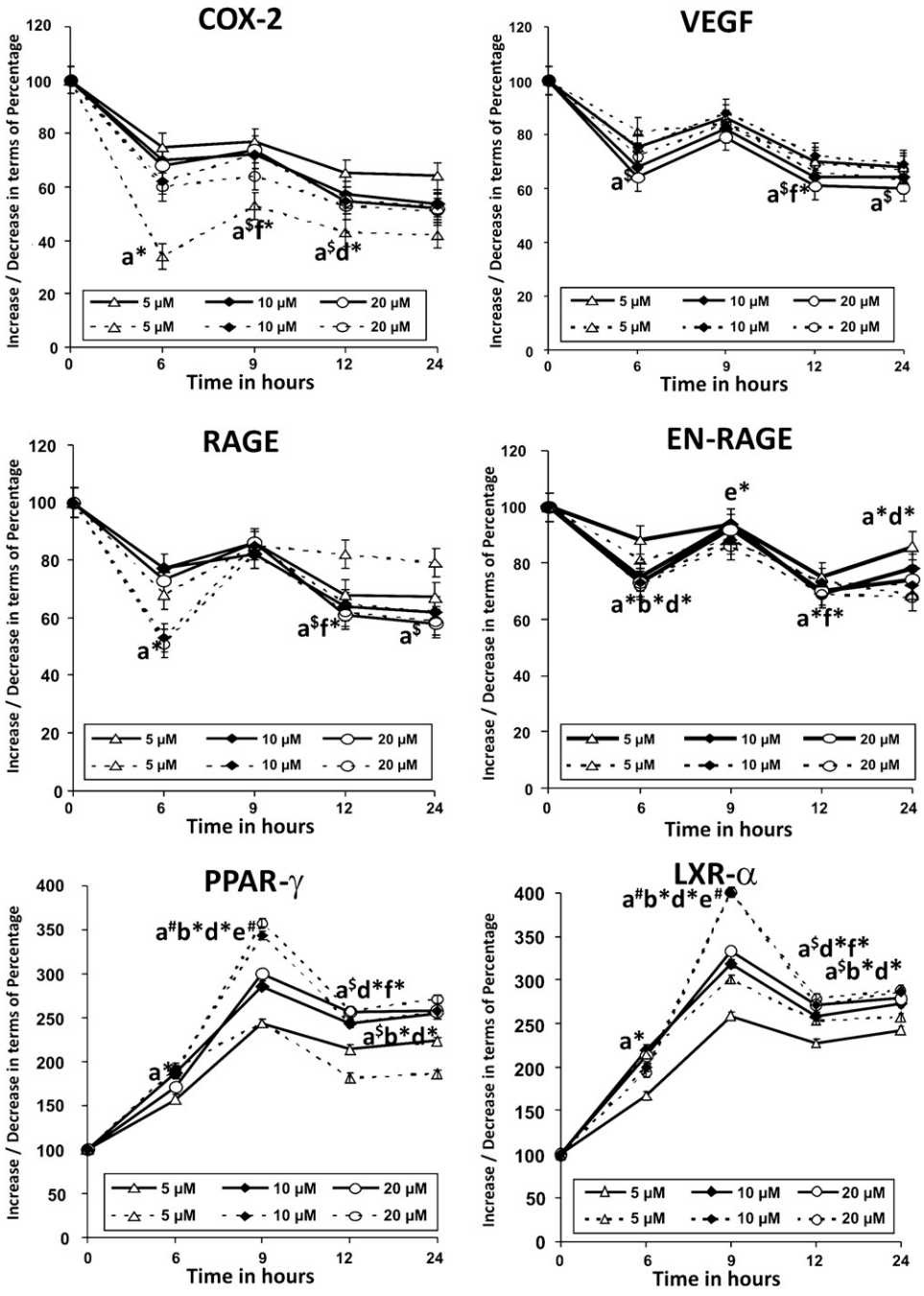
In contrast, a marked increase in mRNA and protein expression of anti-inflammatory genes PPAR-$\gamma$ and LXR-$\alpha$ was observed with similar doses of atorvastatin. Statins have been shown to increase the LXR-$\alpha$ expression in vitro in monocytes, hepatocytes, and leukocytes (21, 23). Grip et al. (20) and Ye et al. (22) reported that atorvastatin activates PPAR-$\gamma$ gene expression and inhibits the production of cytokines, chemokines, and matrix metalloproteinases both in vitro and in vivo. These findings suggest that, besides inflammation, atorvastatin also inhibits other oxidative stress–related molecular species at the sites of inflammation. It is well documented that the endometriotic implants are characterized by an environment rich in pro-oxidant, proinflammatory, and inflammatory mediators (3, 6, 24). Thus, our data strongly suggest that atorvastatin therapy may prove to be of benefit in the treatment of endometriosis.

Recently, Oktem et al. (41) observed that a high dose of atorvastatin for 21 days significantly reduced the size of experimentally induced endometriotic implants and VEGF levels in the peritoneal fluid in rats. On the basis of these findings, we can extrapolate that, in humans, atorvastatin treatment in a higher dose (80 mg/d) may be sufficient to induce the angiostatic effects of atorvastatin. Physiologic levels of atorvastatin in humans range between 0.002 and 0.2 mmol/L (43). Atorvastatin is usually prescribed in humans in doses of 10 to 80 mg/d to lower the serum lipids as deemed appropriate; however, atorvastatin at 80 mg/d could be viewed as an upper limit for those subjects with endometriosis who do not have hyperlipidemia, as there were in our study (data not shown).

Atorvastatin significantly inhibited proliferation and differentiation of endometriotic stromal cells in subjects with endometriosis, down-regulated inflammatory gene expression, and up-regulated anti-inflammatory genes, which in our view could be visualized of therapeutic importance in the course of endometriosis disease. Though no effect of the drug could be observed for 17$\beta$-$E_2$ levels in our study, Honjo et al. (44) and Böhm et al. (45) reported that pravastatin treatment did not alter steroid levels or gonadotropins in doses that significantly reduced total and low-density–lipoprotein cholesterol. This effect also can be viewed as favorable in subjects with endometriosis because most of the therapies currently used to treat endometriosis act by altering the hormonal state of the individual (29) and produce chronic anovulation, endometrial decidualization, or atrophy (46, 47).

## FIGURE 2

Time- and dose-dependent effects of atorvastatin on LPS-induced mRNA expression of test genes in endometriotic stromal cells of group 1 (n = 15) and 2 (n = 10) subjects. ——, Group 1, – – –, group 2. a = Atorvastatin-treated versus LPS-induced mRNA expression; b = 5 μmol/L versus 10 μmol/L; c = 10 μmol/L versus 20 μmol/L; d = 5 μmol/L versus 20 μmol/L; e = 6 hours versus 9 hours; f = 9 hours versus 12 hours; g = 12 hours versus 24 hours. *$P < .05$; $^{\$}P < .01$; $^{\#}P < .001$.

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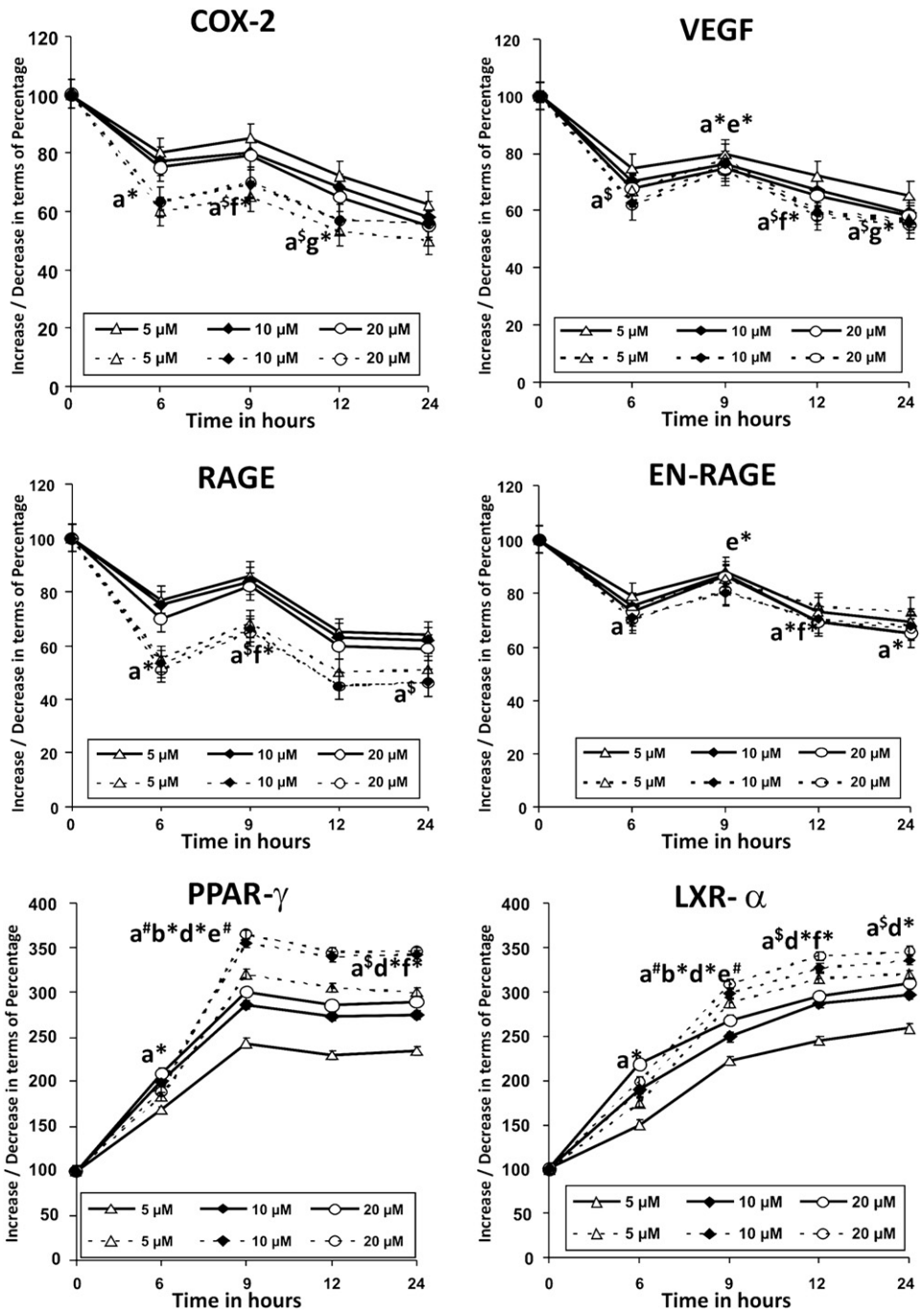
Because the clinical profile of endometriosis is typical of inflammatory disease, therapy with anti-inflammatory drugs is conceivable. Our data clearly demonstrate that atorvastatin has an inherent ability to restore anti-inflammatory status and to counteract the neoangiogenic process in vitro. Also, atorvastatin was able to regulate the proliferation of endometriotic stromal cells, which is of critical importance particularly in conditions with recurrence of endometrioma. Hence, the results of our in vitro study provide new insights for the therapeutic implications of atorvastatin in conditions such as endometriosis. Our findings also strengthen and justify the use

## FIGURE 3

Time- and dose-dependent effects of atorvastatin on LPS-induced protein expression of test genes in endometriotic stromal cells of group 1 (n = 15) and 2 (n = 10) subjects. ——, Group 1;– – –, group 2; a = atorvastatin-treated versus LPS-induced protein expression; b = 5 μmol/L versus 10 μmol/L; c = 10 μmol/L versus 20 μmol/L; d = 5 μmol/L versus 20 μmol/L; e = 6 hours versus 9 hours; f = 9 hours versus 12 hours; g = 12 hours versus 24 hours. *$P<.05$; $^{\$}P<.01$; $^{\#}P<.001$.

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of atorvastatin in clinical trials in humans in the near future as a new anti-inflammatory and antiangiogenic approach or as an adjunct therapy to prevent the progression and development of this disease.

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## SUPPLEMENTARY TABLE 1

**Primer characteristics and program for reverse transcriptase–polymerase chain reaction.**

| S. no. | Gene | Primer pair and polymerase chain reaction program | Cycles |
|---|---|---|---|
| 1 | $\beta$-Actin (257 bp) | Forward: 5′-CATGTACGTTGCTATCCAGGC-3′<br>Reverse: 5′-CTCCTTAATGTCACGCACGAT-3′<br>i. 94°C 5 min (initial denaturation)<br>ii. 94°C 45 s<br>58°C 30 s<br>72°C 45 s<br>iii. 72°C 10 min (final extension) | 30 |
| 2 | COX-2 (305 bp) | Forward: 5′-ATGACAAAACTTGAAGAG-3′<br>Reverse: 5′-CTACTCTTTGTGGGTGTG-3′<br>i. 94°C 5 min (initial denaturation)<br>ii. 94°C 30 s<br>60°C 30 s<br>72°C 1 min<br>iii. 72°C 10 min (final extension) | 35 |
| 3 | VEGF (263 bp) | Forward: 5′-ATGACAAAACTTGAAGAG-3′<br>Reverse: 5′-CTACTCTTTGTGGGTGTG-3′<br>i. 94°C 5 min (initial denaturation)<br>ii. 94°C 30 s<br>60°C 30 s<br>72°C 1 min<br>iii. 72°C 10 min (final extension) | 35 |
| 4 | RAGE (201 bp) | Forward: 5′-CAGGAATGGAAAGGAGACCA-3′<br>Reverse: 5′-CCCTTCTCATTAGGCACCAG-3′<br>i. 94°C 4 min (initial denaturation)<br>ii. 94°C 40 s<br>56°C 50 s<br>72°C 50 s<br>iii. 72°C 7 min (final extension) | 40 |
| 5 | EN-RAGE (280 bp) | Forward: 5′-ATGACAAAACTTGAAGAG-3′<br>Reverse: 5′-CTACTCTTTGTGGGTGTG-3′<br>i. 94°C 5 min (initial denaturation)<br>ii. 94°C 30 s<br>60°C 30 s<br>72°C 1 min<br>iii. 72°C 10 min (final extension) | 40 |
| 6 | PPAR-$\gamma$ (297 bp) | Forward: 5′-TCTCTCCGTAATGGAAGACC-3′<br>Reverse: 5′-GCATTATGAGACATCCCCAC-3′<br>i. 94°C 5 min (initial denaturation)<br>ii. 94°C 30 s<br>62°C 30 s<br>72°C 1 min<br>iii. 72°C 10 min (final extension) | 35 |
| 7 | LXR-$\alpha$ (564 bp) | Forward: 5′-ATGGACACCTACATGCGTC-3′<br>Reverse: 5′-ATGGTGGATGGAGACGTAG-3′<br>i. 94°C 4 min (initial denaturation)<br>ii. 94°C 45 s<br>62°C 45 s<br>72°C 45 s<br>iii. 72°C 5 min (final extension) | 40 |

*Note:* bp = base pairs.

*Sharma. In vitro effects of atorvastatin in endometriosis. Fertil Steril 2010.*