Progesterone, but Not Medroxyprogesterone, Inhibits Vascular Cell Adhesion Molecule-1 Expression in Human Vascular Endothelial Cells

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Abstract—It has been shown that ovarian steroid hormones can reduce the incidence of cardiovascular disease in postmenopausal women. As hormone replacement therapy for postmenopausal women, progestins are added to estrogen to eliminate the increased risk of endometrial cancer. However, the effects of progestins on the atherogenic process have not been well understood. In the present study, we examined the effects of progestins on the expression of vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs). Immunocytochemical analysis revealed the presence of progesterone receptors in HUVECs. Progesterone clearly inhibited tumor necrosis factor-α-activated expression of VCAM-1 protein and its mRNA in HUVECs. Synthetic progesterone receptor agonist R5020 also inhibited the tumor necrosis factor-α-activated VCAM-1 expression, whereas medroxyprogesterone acetate (MPA) failed to do so. Electrophoretic mobility shift assays demonstrated that progesterone, but not MPA, inhibited DNA binding of the transcription nuclear factor-κB, which is critical for the inducible expression of VCAM-1. Because the expression of VCAM-1 is one of the earliest events that occurs in the atherogenic process, this adhesion molecule might be a target molecule for progesterone on vascular walls. The contrasting effects of progesterone and MPA seem clinically important, inasmuch as MPA is a widely used progestin in the regimen of hormone replacement therapy. (Arterioscler Thromb Vasc Biol. 2001;21:243-248.)

Key Words: progesterone ■ medroxyprogesterone ■ vascular cell adhesion molecule-1 ■ endothelial cells ■ progesterone receptors

Compared with men of similar age and postmenopausal women, premenopausal women are known to be protected against cardiovascular diseases.1-3 Ovarian steroid hormones have been reported to play an important role in the protection against cardiovascular diseases. There are many reports indicating that estrogens have beneficial effects on atherosclerotic vascular diseases in postmenopausal women.4-10 In contrast, the effects of progestins, which are used to eliminate the increased risk of endometrial cancer by estrogen treatment,11 on vascular walls have not been well understood. Recently, Grodstein et al12 have shown that a combination therapy with estrogens and progestins lowered the incidence of cardiovascular disease in postmenopausal women. As hormone replacement therapy for postmenopausal women, progestins are added to estrogens to protect women against cardiovascular diseases.1-3 Ovarian steroid hormones have been reported to play an important role in the protection against cardiovascular diseases. There are many reports indicating that estrogens have beneficial effects on atherosclerotic vascular diseases in postmenopausal women.4-10 In contrast, the effects of progestins, which are used to eliminate the increased risk of endometrial cancer by estrogen treatment,11 on vascular walls have not been well understood. Recently, Grodstein et al12 have shown that a combination therapy with estrogens and progestins lowered the incidence of cardiovascular disease in postmenopausal women compared with therapy with estrogens alone. In addition, it has been shown that in ovariec-tomized baboons, the administration of 17β-estradiol and progesterone together resulted in fewer vascular lesions than the administration of 17β-estradiol alone.13 These results suggest that progestins independently exert antiatherogenic effects.

It has been shown that progesterone induces endothelium-independent relaxation in rabbit coronary arteries14 and also inhibits the induction of platelet calcium responses.15 A recent study by Lee et al16 has clearly shown that progesterone inhibits arterial smooth muscle cell proliferation via downregulating the expression of cyclin A and E. Cheng et al17 have demonstrated the effects of progesterone on macrophages: inhibition of cholesterol ester synthesis and block of glucocorticoid-induced increases in cholesterol ester synthesis. In addition, Miyagawa et al18 have shown that progesterone plus 17β-estradiol protects against coronary vasospasm in ovariec-tomized rhesus monkeys. Thus, the studies concerning the effects of progestins on atherogenesis have mainly focused on vascular smooth muscles as well as lipid metabolism. However, to date, there is no evidence of the direct effects of progestins on vascular endothelial cells.

Atherosclerosis is characterized by endothelial cell injury, which in turn leads to the adhesion of mononuclear leukocytes to the endothelium, the initial migration and proliferation of smooth muscle cells, and extracellular matrix deposition.19 Various adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1, E-selectin, and platelet endothelial cell adhesion...
molecule, have been demonstrated to be expressed in atherosclerotic lesions, which might be involved in mononuclear leukocyte adhesion to the vascular endothelium.20–24 Thus, to repress the expression of these adhesion molecules on vascular endothelium may make it a molecular target of antiatherogenic agents. Indeed, it has been recently shown that some activators of the nuclear receptors, such as estrogen receptors25,26 and peroxisome proliferator–activated receptors,27,28 target the adhesion molecules in vascular endothelial cells. In the present investigation, we studied whether progesterone and/or medroxyprogesterone acetate (MPA) influences VCAM-1 expression in cultured vascular endothelial cells.

Methods

Materials and Cell Culture

Promegestone (R5020) was obtained from NEN Life Products. 17α-Hydroxy-11-(4-dimethylaminophenyl)-17-propenyl-4,5-diene-3-one (RU486) was from Biomol. Onapristone (ZK98299) was provided by Schering. All other steroid compounds were purchased from Sigma Chemical Co.

Human umbilical vein endothelial cells (HUVECs, Cascade Bioiogics) were maintained in MCDB131 medium with 10% FCS (IRH Biosciences) and 2 ng/mL basic fibroblast growth factor (Kaken Pharmaceutical) (growth medium). The cells have the characteristics of vascular endothelial cells in terms of cobblestone appearance at confluence and von Willebrand factor expression.

Whole-Cell ELISA

HUVECs were plated onto 96-well collagen-coated dishes in the growth medium. After 24 hours, the culture medium was changed to DMEM–15% gelding horse serum (Sigma) without basic fibroblast growth factor. The cells were pretreated with test compounds or vehicle (0.1% ethanol) for 24 hours and thereafter were stimulated with 20 ng/mL tumor necrosis factor (TNF)-α (Dainippon Pharmaceutical) for 4 hours. ELISA for cell surface VCAM-1 protein was performed as described below. After treatment with 2% paraformaldehyde, the cells were washed twice with 1% BSA in PBS and then incubated for 1 hour with a primary antibody specific to VCAM-1 (Genzyme) diluted 1/1000 in 3% BSA in PBS. Then the cells were washed 4 times and incubated for 1 hour with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham) diluted 1:1000 in 3% BSA in PBS. After the cells were washed, the second antibody binding was detected by reaction of tetramethylbenzidine with H₂O₂ (TMB peroxidase ELISA substrate kit, Bio-Rad). The reaction was stopped by the addition of 25 μL of 8N sulfuric acid, and plates were read on an ELISA reader at an optical density of 450 nm. In parallel, HUVECs were treated as described above. Cell extracts were prepared from these cells in lysis buffer and assayed for protein content by a BCA protein assay reagent kit (Pierce).

Northern Blot Analysis

HUVECs were grown in the growth medium on 10-cm collagen-coated dishes until they were subconfluent. After the culture medium was changed to DMEM–15% gelding horse serum (without basic fibroblast growth factor), the cells were treated with test compounds and/or TNF-α. Total RNA extracted by the acid guanidinium thiocyanate–phenol–chloroform method was electrophoresed in 1% agarose–2% formaldehyde gels. The gels were transferred to nylon membranes (Hybond N+, Amersham), and the membranes were UV-irradiated. A 32P-labeled cDNA probe for human VCAM-1 was prepared by a random priming method (Amersham). Hybridization was carried out by using Quick hybridization buffer (Stratagene).

Nuclear Extraction and EMSA

HUVECs were treated with test compounds and/or TNF-α, as described above. The nuclear extracts were prepared by the method of Schreiber et al.29 An electrophoretic mobility shift assay (EMSA) for nuclear factor (NF)-κB was performed, as described.30 The sequence of double-stranded oligonucleotides used to determine the DNA binding abilities of NF-κB was that derived from the human VCAM-1 gene promoter. The NF-κB consensus sequence is shown in bold face: 5′-CTGCCCTTGGGTTCCTTGAAGGGATTTCCCTCCGCC-3′.

Immunocytochemistry

After HUVECs were treated with 2% paraformaldehyde, the cells were incubated with 0.1 mol/L glycine in PBS and then with 0.1% Triton X-100 in PBS. After they were washed, the cells were incubated with 5% goat serum in PBS for 1 hour and thereafter with antibody against the progesterone receptor (PgR, Santa Cruz) diluted 1:200, followed by incubation with biotinylated anti-rabbit IgG antibody. The next steps were performed with the use of Vectastain ABC kit (Vector Laboratories).

For immunostaining of NF-κB p65 protein, formaldehyde-fixed HUVECs were incubated with antibody against p65 (Santa Cruz) diluted 1:200, followed by incubation with FITC-conjugated secondary antibody (Jackson Immunoresearch). Probes were excited with the 492-nm line of an argon-ion laser. Emitted light was detected at 520 nm.

Statistical Analysis

All values represent mean±SD. When the significant difference was discussed, an unpaired Student t test was used.

Results

PgRs in HUVECs

We examined whether PgRs are expressed in HUVECs. Immunocytochemical analysis using PgR-specific antibody (Figure 1) revealed perinuclear localization of PgRs in HUVECs. When HUVECs were treated with progesterone, the staining was translocated into nuclei. The staining was blocked after preabsorption of anti-PgR antibody with PgR antigen (B and D).

![Figure 1. Expression of PgRs in HUVECs. Immunocytochemistry using PgR-specific antibody revealed perinuclear staining in HUVECs (A). When HUVECs were treated with 10⁻⁹ mol/L progesterone, the staining was localized into nuclei (C). The staining was blocked after preabsorption of anti-PgR antibody with PgR antigen (B and D).](http://atvb.ahajournals.org/)
the TNF-α–induced increase of VCAM-1 protein in a dose-dependent manner (Figure 2). Significant repression was observed at concentrations $\leq 10^{-7}$ mol/L. At $10^{-5}$ mol/L, progesterone inhibited TNF-α–induced stimulation of VCAM-1 levels by 83%. However, progesterone did not show any significant change of unstimulated VCAM-1 levels (Figure 2). The synthetic PgR agonist R5020 ($10^{-5}$ mol/L) inhibited the TNF-α–induced increase of VCAM-1 by 44% (Figure 3). By contrast, MPA showed no effect on the TNF-α–induced stimulation of VCAM-1 levels. Neither 17α-hydroxyprogesterone, pregnenolone, 17α-hydroxy-pregnenolone, nor 5β-pregnane-3,20-dione demonstrated inhibition of the TNF-α–induced VCAM-1 expression (Figure 3). Progesterone-mediated inhibition of VCAM-1 expression was not blocked by PgR antagonists RU486 and ZK98299 (please see Figure II, published online and available at http://atvb.ahajournals.org). As shown in Figure III (published online and available at http://atvb.ahajournals.org), 17β-estradiol also reduced the TNF-α–induced VCAM-1 levels, but it failed to augment progesterone-mediated inhibition of the VCAM-1 expression.

Next, to examine the effects of progestins on the levels of VCAM-1 mRNA in HUVECs, Northern blot analyses were performed. As shown in Figure 4, TNF-α increased VCAM-1 mRNA accumulation. Treatment of the cells with progesterone significantly reduced the TNF-α–induced accumulation of VCAM-1 mRNA. By contrast, MPA failed to reduce the VCAM-1 mRNA accumulation.

**Effects of Progestins on Activation of NF-κB in HUVECs**

TNF-α–induced activation of VCAM-1 transcription in vascular endothelial cells has been shown to depend, at least in part, on the activation of the transcription factor NF-κB. Thus, we examined the effect of progestins on TNF-α–induced activation of NF-κB in HUVECs. EMSA with the nuclear extracts from HUVECs and the oligonucleotide probe containing NF-κB binding sites in the human VCAM-1 gene promoter showed that TNF-α enhanced 2 of the gel-retarded bands (Figure 5, lanes 1 and 2). The gel-retarded bands were specific for NF-κB, inasmuch as it disappeared in the presence of the NF-κB-specific antisense probe.
HUVECs with progesterone or R5020 repressed the TNF-α band consists of p65-p65 homodimers and that the lower bands (Figure 5, lane 7). These results indicate that the upper antibodies almost completely supershifted the gel-retarded heads indicate NF-κB proteins, subcellular localization of NF-κB p65 was investigated. As demonstrated in Figure 6,NF-κB facilitated the translocation of the p65 protein from the cytoplasm into nuclei. Progesterone failed to prevent the TNF-α–induced translocation of the p65 protein. Treatment with progesterone alone did not show any effect on the localization of the p65 protein.

Discussion
In the present investigation, we for the first time demonstrated that progesterone significantly repressed levels of cell surface VCAM-1 protein enhanced by TNF-α in human vascular endothelial cells. The synthetic PgR agonist R5020 also inhibited the TNF-α–induced VCAM-1 expression. By contrast, 17α-hydroxyprogesterone, pregnenolone, 17α-hydroxyprogrenolone, and 5β-pregn-3,20-dione, which can activate the pregnane X receptor (PXR) as does progesterone,34 did not show any effect on the TNF-α–induced VCAM-1 expression. Immunocytochemical study revealed the expression of PgRs in HUVECs. Thus, the inhibitory effect of progesterone on VCAM-1 expression is suggested to be mediated via PgR but not the pregnane X receptor, although the involvement of other receptors cannot be completely ruled out. In this relation, PgR has been shown to be present in canine vascular tissue,35 baboon and human aorta,36,37 and human arterial smooth muscle cells.16

Progesterone significantly exerts its inhibitory effect at a concentration of 10−7 mol/L, although higher concentrations are necessary to induce more profound inhibition of VCAM-1 expression. The effective concentration of progesterone (≥10−7 mol/L) seems higher than its physiological concentration in human sera. Therefore, a pharmacological concentration of progesterone may be needed to exert the inhibitory effect on adhesion molecule expression. Alternatively, it is also possible that a physiological concentration of progesterone is enough to demonstrate such an effect in vivo, because of intact vascular microenvironment or the prolonged exposure to progesterone. In similar instances, other studies on VCAM-1 regulation by estrogens and glucocorticoids25,26 have also noted that their inhibitory effects were more evident at concentrations that were higher than their physiological concentrations.

VCAM-1 is known to play an important role in mediating mononuclear leukocyte-selective adhesion to vascular endothelium.20,38 This adhesion molecule is expressed in the vascular lesions in early atherosclerosis,21 and its soluble form was elevated in sera from patients with early atherosclerosis,39,40 indicating that VCAM-1 is one of the key molecules involved in the atherogenic process. Therefore, the progesterone-induced repression of VCAM-1 expression in
vascular endothelial cells is suggested to be a molecular mechanism of the antiatherogenic action of progesterone.

In the present study, MPA, a synthetic progestin, was different from progesterone in that it had no effect on TNF-α–induced VCAM-1 expression. A recent observation has also shown the differential effect of MPA from progesterone. In that study, progesterone plus 17β-estradiol protected coronary vasospasm in ovariectomized rhesus monkeys, whereas MPA plus 17β-estradiol failed to protect. Such contrasting effects of progesterone and MPA are clinically important issues, inasmuch as MPA is now a widely used progestin in the regimen of hormone replacement therapy. In this relation, several studies have demonstrated unfavorable effects of MPA on lipid metabolism and atherosclerosis.

The present study showed that TNF-α–induced VCAM-1 mRNA accumulation was also reduced by progesterone. Thus, the progesterone-induced repression of VCAM-1 is indicated to occur at pretranslational levels. Because there is no evidence that progesterone response elements exist on the VCAM-1 gene promoter, it is unlikely that progesterone-bound PgR directly interacts with the 5'–flanking region of the VCAM-1 gene. Rather, progesterone exerts its effect by an indirect mechanism. TNF-α–induced transcription of the VCAM-1 gene in vascular endothelial cells is known to be dependent, at least in part, on the activation of NF-κB transcription factors. In our experiments, progesterone as well as R5020 clearly suppressed TNF-α–activated NF-κB binding to its specific DNA binding elements. In contrast, MPA had no inhibition of the NF-κB binding. Thus, the differential effects of these progestins on the NF-κB binding are correlated with those on VCAM-1 expression. Therefore, the progesterone-induced repression of VCAM-1 expression is mediated at least via interference with the NF-κB activity. The present study has demonstrated that in HUVECs TNF-α–activated NF-κB proteins consist mainly of p65 (RelA) and p50 (NFκB1). Immunocytochemical analysis revealed that progesterone did not block TNF-α–stimulated translocation of NF-κB p65 protein from cytoplasm to the nuclei, indicating that the progesterone exerts its effect mainly at steps after the nuclear translocation of NF-κB. Thus, progesterone is suggested to demonstrate its inhibitory effect on VCAM-1 expression by transpressing the NF-κB activity. Such a different mechanism from PgR-mediated transactivation of the target genes might be the effective concentration of progesterone appears to be higher. It is undefined how progesterone and R5020, but not MPA, inhibit NF-κB binding to its specific DNA binding elements of the VCAM-1 gene promoter. Much evidence has accumulated indicating that several coactivators, such as cAMP response element–binding protein/p300, steroid receptor coactivator-1/nuclear receptor coactivator-1, and transcription intermediary factor 2/glucocorticoid receptor–interacting protein 1/nuclear receptor coactivator-2, are involved in the signaling of various nuclear receptors, including PgR. Among these coactivators, cAMP response element–binding protein/p300 and steroid receptor coactivator-1 have been shown to interact with NF-κB, enhancing its transcriptional activity. Thus, it is possible that progesterone- or R5020-bound PgR, but not MPA-bound PgR, may interfere with the NF-κB activity by squelching such coactivators. Another possibility is that PgR directly interacts with NF-κB in a ligand-specific manner, followed by the reduction of NF-κB transcriptional activity. In this context, cotransfection experiments showed that the concomitant expression of PgR and NF-κB results in mutual antagonism of the transcriptional activities between PgR and NF-κB. In the present study, combined treatment with 17β-estradiol and progesterone did not demonstrate any additive inhibition on VCAM-1 expression, suggesting that estrogen receptors can exhibit an inhibitory effect on VCAM-1 expression by sharing the same molecular targets as PgR. In addition, the present study showed that RU486 and ZK98299 failed to antagonize the progesterone-mediated inhibition of VCAM-1 expression. These results are not surprising, inasmuch as these “agonists” have been demonstrated to repress NF-κB–evoked transcriptional activity as the “agonist” in transfection systems.

In conclusion, we have shown that progesterone, but not MPA, reduces TNF-α–enhanced VCAM-1 expression in vascular endothelial cells. The effect of progesterone may be mediated via the inhibition of binding of NF-κB to the specific sequence of the VCAM-1 gene promoter. To our knowledge, this is the first study to demonstrate that progesterone inhibits the gene expression in which NF-κB is involved. In view of these observations, we believe that the inhibitory effect on VCAM-1 expression in vascular endothelial cells should be added to the lists of biological actions of progesterone on vascular walls.

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Figure II

![Graph showing YCAM-1 (OD 50/mg protein) against Progesterone (M) with control, RU486, and ZK98299 treatments at different concentrations.](image-url)
Figure III
Figure I