Certain Progestins Prevent the Enhancing Effect of 17β-Estradiol on NO-Mediated Inhibition of Platelet Aggregation by Endothelial Cells

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Objectives—Estro-progestin treatments have been associated with an increased risk of thromboembolic events in postmenopausal women. This study examined whether progestins affect the stimulatory effect of estrogens on the endothelial formation of nitric oxide (NO), a potent antithrombotic factor.

Methods and Results—Experiments were performed with human endothelial cells. Endothelial NO synthase (eNOS) and GTP cyclohydrolase I (GTPCH I) mRNA expression was assessed by RT-PCR, eNOS protein by Western blotting, NO formation by electron spin resonance spectroscopy, and platelet aggregation by an aggregometer. Medroxyprogesterone acetate (MPA), progesterone, levonorgestrel, and nomegestrol acetate prevented the 17β-estradiol (17β-E)–induced expression of eNOS mRNA and protein. MPA and progesterone reduced the 17β-E–induced formation of NO and potentiation of the inhibitory effect of endothelial cells on platelet aggregation whereas levonorgestrel and nomegestrol acetate were without effect. Moreover, MPA and progesterone prevented the 17β-E–induced expression of GTPCH I mRNA. Mifepristone, a glucocorticoid and progestogen receptor antagonist, and L-sepiapterin prevented the inhibitory effect of MPA and progesterone on platelet aggregation.

Conclusions—Certain progestins, including MPA, attenuate the 17β-E–induced NO-mediated inhibition of platelet aggregation through preventing both eNOS and GTPCH I expression most likely via activation of glucocorticoid receptors. (Arterioscler Thromb Vasc Biol. 2009;29:586-593.)

Key Words: 17β-estradiol ■ progestins ■ endothelial nitric oxide synthase ■ GTP cyclohydrolase ■ thrombosis

Estrogens have a beneficial influence on the cardiovascular system. The protective effect includes an improvement of the lipid profile and an inhibition of the expression of proatherosclerotic molecules such as vascular cell adhesion molecule-1, monocyte chemottractant protein-1, endothelin-1, and NADPH oxidase. The beneficial effect of estrogens might also be attributable to their direct action on blood vessels. Indeed, estrogens activate the endothelial NO synthase (eNOS) through nongenomic mechanisms involving the rapid activation of the ERK/MAP kinase and the PI3-kinase/Akt pathway. This rapid activation of eNOS is mediated by both estrogen receptors, ER-α and ER-β. In addition, chronic treatment with 17β-estradiol (17β-E) potentiated endothelium-dependent relaxations to acetylcholine in the rabbit femoral artery and in the guinea pig coronary circulation. Such long-term effects have been explained by an upregulation of the expression of eNOS by 17β-E by increasing gene transcription and mRNA stability. An estrogen-induced upregulation of eNOS expression has been observed in vivo in some, but not all studies.

In the Women’s Health Initiative, an increased relative risk of cardiovascular diseases has been observed in postmenopausal women receiving conjugated equine estrogens (CEE) and MPA whereas no such effect was observed in women receiving only CEE, suggesting that the progestin might contribute to the adverse effects on the cardiovascular system.

See accompanying article on page 441

In addition, MPA alone has been shown to reduce the inhibitory effect of endothelial cells on platelet aggregation by downregulating the expression of eNOS via activation of glucocorticoid receptors. In contrast, levonorgestrel and nomegestrol acetate, which are devoid of partial glucocorticoid activity, did not have such an effect. Therefore, the aim of the present study was to determine whether progestins inhibit the beneficial effect of estrogens on the formation of NO, a potent vasoprotective and antithrombotic factor, in human endothelial cells and, if so, to determine the underlying mechanisms.
Methods

Cell Culture
Endothelial cells (HUVECs) were isolated from human umbilical veins as described previously. Cells were cultured in MCDB 131 without phenol red containing 2 mmol/L ultraglutamine, 100 U/L penicillin, 100 mg/L streptomycin supplemented with 10% FCS. Confluent cells were used at first passage and were serum-deprived for 24 hours before experiments.

Hormonal Treatments
At confluence, HUVECs were serum-deprived for a 24-hour period before being exposed to either solvent (0.01% DMSO), a progestin or hydrocortisone alone or in combination with 17β-E. Two categories of progestins have been studied, those with partial glucocorticoid activity such as progesterone and medroxyprogesterone acetate (MPA), and those without partial glucocorticoid activity such as levonorgestrel (LNG) and nomegestrol acetate (NOMAC). Most experiments have been performed with progestins used at concentrations up to 0.1 μmol/L, which can be reached in the plasma of postmenopausal women using hormonal replacement therapy.

Real-Time Polymerase Chain Reaction
Total RNA was isolated from HUVECs using RNAeasy Micro kit (QIAGEN). cDNA was synthesized from total RNA using iScript cDNA Synthesis kit (BIORAD), and PCR amplification was performed using IQ SYBR Green Supermix (BIORAD). The specific primers were as follows: eNOS sense, 5’-GTC GAC ACC AGG AAC ACC 3’; eNOS antisense, 5’-GCA CTC CTC TCG CCA TCA C-3’; GTPCH I sense, 5’-GCC ATG CAG TCC TTC ACC AAA-3’; GTPCH I antisense, 5’-AGG CCT TCC TGA TCT CTA CA-3’. The control housekeeping gene was human GAPDH. Relative quantitation was determined by standard 2(−ΔΔCt) calculations.

Western Blot Analysis
Total proteins (15 to 20 μg) were subjected to SDS-PAGE (8%) and blotted on PVDF membranes. Immunodetection was carried out using an antibody directed against eNOS (BD Biosciences) or β-tubulin (Sigma) and enhanced chemiluminescence (Amersham).

Determination of NO Formation by Electron Spin Resonance Spectroscopy
Determination of NO formation was assessed in endothelial cells by electron spin resonance spectroscopy (ESR) after formation of [Fe(II)NO(DETC)2], a paramagnetic diethyldithiocarbamate iron complex with NO. The ESR methodology was used as reported previously.

Platelet Aggregation Studies
Human washed platelet suspensions were prepared as previously described. Suspensions of washed platelets (450 μL, 3.10⁸ platelets/mL) were incubated for 2 minutes in a Chronolog 490 aggregometer (Haverton) with continuous stirring at 1000 rpm before addition of a submaximal concentration of thrombin (0.07 U/mL). HUVECs were cultured on cytodex-3 beads, and when they reached confluence they were exposed to either solvent or a compound for 24 hours. In some experiments, HUVECs were treated with either mifepristone (10 μmol/L) for 30 minutes before addition of a progestin for 24 hours or L-sepiapterin (30 μmol/L) for the last 6 hours of the 24-hour treatment period. Thereafter, beads covered with HUVECs were let to settled down in the culture flask and a volume of 30 to 50 μL of beads (about 500 cells) was taken and added to platelet suspensions 1 minute before addition of thrombin.

Statistical Analysis
All data are expressed as mean±SEM. Statistical analysis of the data were performed using Friedman test. A value of P<0.05 was considered statistically significant.

Results

Progestins Impair 17β-Estradiol-Induced Expression of eNOS
Exposure of HUVECs to 17β-E (10 nmol/L) significantly increased eNOS mRNA and protein levels (Figure 1 and 2). Levonorgestrel, progesterone, nomegestrol acetate, and MPA (all tested at 0.1 μmol/L) alone did not affect the basal eNOS mRNA level, but prevented the stimulatory effect of 17β-E (Figure 1A and 1B). Levonorgestrel, progesterone, nomegestrol acetate, and MPA also abolished the 17β-E-induced expression of eNOS protein (Figure 2). The inhibitory effect was mimicked by the glucocorticoid hydrocortisone (supplemental Figure 1). In addition, the inhibitory effect of progestins and hydrocortisone was concentration-dependent with a significant effect observed at concentrations as low as 1 nmol/L for progesterone, levonorgestrel, and hydrocortisone, and 10 nmol/L for nomegestrol acetate (Figure 2 and supplemental Figure 1).

Effect of Progestins on 17β-Estradiol-Induced Formation of NO
Exposure of HUVECs to 17β-E (10 nmol/L) for 24 hours increased by about 54% the formation of NO as assessed by ESR (Figure 3). The stimulatory effect of 17β-E was significantly reduced by progesterone and MPA by about 61% and...
76%, respectively (Figure 3). In contrast, nomegestrol acetate reduced slightly but not significantly the formation of NO and levonorgestrel was without effect (Figure 3). In addition, basal NO formation was not affected by either progestin alone (Figure 3).

**Certain Progestins Impair the 17β-Estradiol–Induced Potentiation of the Inhibition of Platelet Aggregation by Endothelial Cells**

Thrombin (0.07 U/mL) caused submaximal and irreversible aggregation of washed human platelet suspensions within 4 to 5 minutes (Figures 4 and 5 and supplemental Figure II). The stimulatory effect of thrombin was significantly reduced by about 30% when HUVECs (about 500 cells) were added to the platelet suspension 1 minute before their activation with thrombin (Figures 4 and 5 and supplemental Figure II). In contrast, addition of HUVECs, which had been pretreated with Nω-nitro-l-arginine (300 μmol/L) for 30 minutes, to the platelet suspension did not affect thrombin-induced platelet aggregation (data not shown). Treatment of HUVECs with 17β-E (10 nmol/L) for 24 hours potentiated the antiaggregatory effect of the cells (Figure 4A). The potentiating effect of 17β-E was abolished by progesterone and MPA, but not by levonorgestrel or nomegestrol acetate (Figure 4B and 4C). The addition of either conditioned culture medium (without beads covered with HUVECs but containing the studied hormone) or freshly prepared culture medium containing the studied hormone at the same concentration affected only minimally platelet aggregation (data not shown). The inhibitory effect of progesterone and MPA was prevented by treating the HUVECs with...
either L-sepiapterin, which produces tetrahydrobiopterin (BH$_4$) via the salvage pathway (Figure 5A and 5B and supplemental Figure IIA) or mifepristone, an antagonist of the progesterone and glucocorticoid receptor (Figure 5C and D and supplemental Figure IIB). Addition of conditioned medium alone from either 17β-H9252-E-, MPA-, progesterone-, L-sepiapterin-, or mifepristone-treated HUVECs did not affect platelet aggregation (data not shown).

Progestins Prevent the Stimulatory Effect of 17β-Estradiol on the Expression of GTP Cyclohydrolase I

Exposure of HUVECs to 17β-E (10 nmol/L) significantly increased the GTPCH I mRNA level, and this effect attained about 84% after a 6-hour treatment period (Figure 6). A small but significant increase of the level of GTPCH I mRNA was also observed with levonorgestrel (35%) and nomegestrol acetate (42%) alone, whereas progesterone and MPA did not have such an effect (Figure 6). Moreover, progesterone and MPA abolished the 17β-E–induced increase in GTPCH I mRNA expression whereas levonorgestrel and nomegestrol acetate had only minor effects (Figure 6).

Discussion

The present findings indicate that all progestins investigated significantly blunt 17β-E-stimulated expression of eNOS mRNA and protein in endothelial cells. In the case of progesterone and MPA, 17β-E–induced formation of NO was also reduced whereas this was preserved in the case of levonorgestrel and nomegestrol acetate despite a similar inhibitory effect on eNOS expression. As an important functional consequence, progesterone and MPA abolished the ability of 17β-E to potentiate the NO-mediated inhibition of platelet aggregation by endothelial cells. Thus, certain progestins including MPA are able to prevent the protective...
effect of estrogens on blood vessels by blunting endothelial NO formation potentially leading to an amplification of prothrombotic responses.

Consistent with previous investigations, 11–13 17\beta-E upregulated the expression of eNOS at both the mRNA and protein level leading to a subsequent increase in NO synthesis. Gene transcription may result from the interaction of estrogen receptors either directly or indirectly after interaction with other transcription factors such as Sp1,27 with the eNOS promoter. Moreover, 17\beta-E has been shown to stabilize eNOS mRNA.28 Alternatively, estrogens may also increase the bioavailability of NO in endothelial cells by lowering the expression of NADPH oxidase and, hence, the degradation of NO by NADPH oxidase-derived superoxide anions.5 The present findings indicate that progestins, including levonorgestrel, nomegestrol acetate, progesterone, and MPA, prevented the stimulatory effect of 17\beta-E on the expression of eNOS mRNA and protein. A similar inhibition of 17\beta-E-induced eNOS protein expression was observed with hydrocortisone, a glucocorticoid; such an effect may be mediated by the binding of glucocorticoid receptors to the newly identified glucocorticoid response element in the eNOS promoter region.29 Moreover, the progestin-induced inhibition of 17\beta-E–induced eNOS expression was observed at concentrations as low as 1 nmol/L progesterone, MPA, or levonorgestrel and 10 nmol/L nomegestrol acetate.

During hormonal replacement therapy or contraception, peak serum concentrations of MPA are about 5 nmol/L after intake of 2 mg 17\beta-E and 5 mg MPA,22 mean serum concentrations of progesterone are about 10 nmol/L after use of a vaginal ring delivering estradiol 160 \mu g/d and progesterone 20 mg/d,23 and mean peak plasma concentrations of levonorgestrel are about 40 nmol/L after intake of 0.75 mg levonorgestrel.30 Thus, the inhibitory effect of progestins on 17\beta-E–induced expression of eNOS is observed at concentrations that are likely to be of clinical significance. The present findings also indicate that progestins alone do not affect the expression of eNOS. In contrast, downregulation of eNOS expression has been observed previously in response to progesterone and MPA whereas levonorgestrel and nomeges-
control acetate were without effect.\textsuperscript{18} Such a difference most likely stems from the fact that basal eNOS levels were much lower in the present study as compared to the previous one, possibly as a consequence of the prolonged exposure of the endothelial cells to serum-free medium before treatment (24 hours versus 6 hours).

As expected, the inhibitory effect of progesterone and MPA on the 17\textbeta-E–induced expression of eNOS mRNA and protein was associated with a blunted formation of NO in response to 17\textbeta-E. In contrast, the 17\textbeta-E–induced formation of NO was not affected by levonorgestrel and nomegestrol acetate despite a similar inhibitory effect on estrogen-induced eNOS expression. The preserved estrogen-induced formation of NO suggests that levonorgestrel and nomegestrol acetate enhance the activity of eNOS or the bioavailability of NO.

Estrogens activate eNOS predominantly via the membrane-associated ER\textalpha and also to some extent through ER-\beta by mechanisms involving several signal transduction pathways. Ligand engagement of ER\alpha initiates its association with the p85a subunit of phosphoinositol 3-kinase and c-Src, leading to Akt activation.\textsuperscript{31,32} This event promotes eNOS phosphorylation at Serine 1177,\textsuperscript{7} a key event that stimulates enzyme activation\textsuperscript{33,34} and enhances its sensitivity to calcium.\textsuperscript{35} ER\textalpha-mediated eNOS activation also involves the mitogen-activated protein kinase pathway and the AMP-activated protein kinase via the promotion of eNOS interaction with heat shock protein 90.\textsuperscript{6,36} Alternatively, estrogens may activate eNOS by enhancing the formation of BH\textsubscript{4}, an essential cofactor of eNOS.\textsuperscript{37} BH\textsubscript{4} levels are predominantly regulated by de novo synthesis, in which GTPCH I is the rate-limiting enzyme.\textsuperscript{38}

The present findings indicate that 17\textbeta-E increased GTPCH I mRNA levels in HUVECs by about 80% after 6 hours. Previous studies have also shown that long-term administration of estrogen to ovariectomized rats increases GTPCH I mRNA levels in the brain\textsuperscript{39} as well as the vascular availability of BH\textsubscript{4}.\textsuperscript{40} In addition, 17\textbeta-E upregulates GTPCH I expression in PC12 cells via transcriptional mechanisms.\textsuperscript{41} The present findings revealed that progesterone and MPA prevent the stimulatory effect of 17\textbeta-E on GTPCH I mRNA expression, whereas levonorgestrel and nomegestrol acetate did not have such an effect. In addition, levonorgestrel and nomegestrol acetate but not progesterone and MPA alone slightly but significantly increased the expression of GTPCH I mRNA. Additional experiments have been performed to study the expression of GTPCH I protein and the endothelial level of BH\textsubscript{4}, however they were not successful because of technical problems. Thus, downregulation of the expression of GTPCH I may contribute to explain the inhibitory effect of progestins on the endothelial formation of NO in response to 17\textbeta-E.

Progestins are a heterogeneous family of steroids that, in addition to their interaction with the cytosolic progesterone receptor, can also bind strongly to other steroid receptors, such as those for androgens, mineralocorticoids, and glucocorticoids, to induce biological responses.\textsuperscript{20,21} The selective inhibition of 17\textbeta-E–induced NO formation and GTPCH I expression by progesterone and MPA but not by levonorgestrel or nomegestrol acetate, despite their similar progestin potency, is not consistent with a major role for the progesterone receptor. Interestingly, progesterone and MPA, the inhibitory progestins, have distinct intrinsic glucocorticoid activity as opposed to levonorgestrel and nomegestrol acetate.\textsuperscript{20,21} In addition, glucocorticoids have been shown to decrease the expression of GTPCH I in isolated arteries leading to an impaired endothelium-dependent relaxation.\textsuperscript{42} Moreover, chronic intake of dexamethasone caused marked hypertension in rats, which was associated with an impaired endothelium-dependent vasodilatation and formation of NO as well as a reduced vascular expression of GTPCH I.\textsuperscript{42,43} Altogether, these previous findings in conjunction with the present ones suggest that the glucocorticoid receptor is involved in the progestin-induced inhibition of endothelial NO formation and GTPCH I expression in response to 17\textbeta-E.

The persistent endothelial formation of NO is a key event in maintaining the endothelial surface thrombo-resistant and blood fluidity. Indeed, NO is a potent antithrombogenic factor by inhibiting platelet adhesion, aggregation, and recruitment, and by preventing the endothelial expression of tissue factor, the physiological activator of the coagulation cascade.\textsuperscript{44–50} The present findings indicate that 17\textbeta-E increases the ability of endothelial cells to inhibit platelet aggregation by enhancing the formation of NO. The beneficial effect of estrogen persists in the presence of levonorgestrel or nomegestrol acetate whereas it is markedly reduced by the exposure of endothelial cells to 17\textbeta-E in combination with progesterone or MPA. The protective effect of estrogen-treated endothelial cells on platelet aggregation in the presence of progesterone

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**Figure 6.** Effect of estro-progestin treatments on GTPCH I mRNA expression. Cells were exposed to levonorgestrel (LNG) and progesterone (PROG) alone or with 17\textbeta-E (A); nomegestrol acetate (NOMAC) and MPA alone or with 17\textbeta-E (B). Data are shown as mean±SEM, n=8 to 9. *P<0.05 vs control and 17\textbeta-E, respectively.

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\textsuperscript{Zerr-Fouineau et al Estradiol, Progestins, eNOS, and Platelet Aggregation}
and MPA was restored in the presence of L-sepiapterin, which produces BH$_2$ via the salvage pathway, and mifepristone, an antagonist of the progesterone and the glucocorticoid receptor. Altogether, the present findings indicate that certain progestins are able to blunt the 17$\beta$E–induced NO-mediated protective effect of endothelial cells on platelet aggregation most likely by activating glucocorticoid receptors with subsequent downregulation of the expression of GTPCH I. In addition, progestins with partial glucocorticoid activity have been shown to markedly potentiate the tissue factor-dependent vascular procoagulant effects of thrombin by increasing the availability of thrombin receptors in the smooth muscle.$^{51}$ The increased prothrombotic response to thrombin$^{51}$ and the decreased endothelial formation of NO (see Zerr-Fouineau et al$^{18}$ and present findings) might contribute to explain the 2-fold increased relative risk for venous thromboembolism or fatal pulmonary embolism in women taking combined low-dose oral contraceptives containing a progestin with partial glucocorticoid activity, such as gestodene and desogestrel, compared to those taking levonorgestrel and norethisterone, compared to those taking levonorgestrel and ethinyloestradiol. Other progestins with partial glucocorticoid activity, such as gestodene and desogestrel, have been shown to be more thrombogenic than levonorgestrel$^{52,53}$.

In summary, the present findings indicate that certain progestins, including MPA, blunt the ability of 17$\beta$E to augment the NO-mediated inhibitory effect of endothelial cells on platelet aggregation. This effect is attributable, at least in part, to the prevention of the stimulatory effect of 17$\beta$E on eNOS and GTPCH I expression most likely via activation of glucocorticoid receptors.

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