Effects of Progesterone and Its Antagonist Mifepristone on Progesterone Receptor A Expression in Human Umbilical Vein Endothelial Cells

Bettina Toth\textsuperscript{a} Christoph Scholz\textsuperscript{b} Robert Ochsenkühn\textsuperscript{a} Sandra Schulze\textsuperscript{b} Christina Kuhn\textsuperscript{b} Klaus Friese\textsuperscript{a, b} Udo Jeschke\textsuperscript{b}

\textsuperscript{a}Department of Obstetrics and Gynecology, Grosshadern, \textsuperscript{b}Department of Obstetrics and Gynecology, Innenstadt, Ludwig Maximilians University, Munich, Germany

Key Words
Human umbilical vein endothelial cells • Progesterone receptor A and B • Progesterone receptor antagonist

Abstract
Effects of female steroid hormones on endothelial cells are gaining increased importance due to several studies on the effects of hormonal treatment on cardiovascular risk. Recent data argue for an improvement of endothelium-derived relaxation and impaired vascular contraction by estradiol, whereas progesterone and testosterone might entail contrary effects. So far, gestagenic influence on endothelial cell physiology is poorly understood. Human umbilical vein endothelial cells (HUVECs) exposed to the female sex hormones estradiol and progesterone show expression of estrogen receptor-\(\beta\) (ER\(\beta\)) and progesterone receptor A (PR-A), and are negative for ER\(\alpha\) and PR-B. The aim of this study was to analyze the expression and stimulation of PR-A and -B in HUVECs after stimulation with progesterone and PR antagonists that are commercially available. PR-B expression or upregulation was abrogated after application of progesterone or antagonists to HUVECs. Expression of PR-A could be significantly upregulated with progesterone and mifepristone. Unexpectedly, stimulation with the progesterone antagonist RU486 (mifepristone) was accomplished by an upregulation of PR-A expression in our study. We conclude that gestagenic effects on HUVECs independent of modulators are mediated via the PR-A.

Introduction
Vascular endothelial cells are involved in the regulation of angiogenesis, inflammatory responses, vascular tone and permeability. Impaired endothelial function leads to increased cardiovascular risk [1]. In females, endothelial dysfunction gradually ensues after the menopause [2], and is associated with disturbed dilatation [3], decline in endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) bioavailability as well as abnormal endothelial morphology [4, 5]. These functional alterations of endothelial cells contribute to the increased risk of cardiovascular diseases seen in postmenopausal women [6]. The incidence of coronary heart disease (CHD) in premenopausal women is significantly lower...
than in age-matched men with similar risk profiles and increases after menopause [7–9].

With regard to hormonal treatment (HT) in postmenopausal women, progesterone is generally co-administered with estrogen to prevent endometrial cancer by opposing the proliferative effect of estrogens. So far, the influence of the administered progestagens on cardiovascular function and development of atherosclerosis remains controversial [10, 11]. However, HT has been regarded as an effective tool to protect postmenopausal women from CHD [12]. Until now, major randomized clinical trials have failed to confirm the cardiovascular advantages of HT [13].

The Heart and Estrogen/Progestin Replacement Study showed that the co-administration of medroxyprogesterone acetate (MPA) with conjugated equine estrogen (CEE) did not reduce the rate of events in postmenopausal women with established CHD, yet the treatment did increase the rate of thromboembolic events and gallbladder disease [14–18].

The Women’s Health Initiative trial showed that HT combined with CEE and MPA was associated with a non-significant increase in CHD in postmenopausal women, whereas women in the sister cohort, receiving CEE alone, showed a nonsignificant decrease in coronary events, along with a significant reduction in a composite outcome of CHD events in younger women [19, 20].

However, progesterone or other synthetic progestins have variable influences on endothelial function. For example, natural progesterone increases endothelial NO production, whereas MPA is devoid of such action [21]. In nonhuman primates, MPA has been shown to interfere with the atheroprotective effects of estrogens, which was not encountered with natural progesterone [22, 23]. In support of these observations, discrepant effects of progestins have also been described in other tissues [24].

A diversity of progesterone receptor (PR) activators and inhibitors exists with different potential to bind to PR-A, PR-B or both. In our study, we analyzed the expression of PR-A and PR-B in human umbilical vein endothelial cells (HUVECs) after stimulation with progesterone and its antagonist mifepristone with specific monoclonal antibodies by immunocytochemistry.

**Material and Methods**

**Cell Culture**

HUVECs were obtained from Promocell (Heidelberg, Germany) at passage 2 or 3. The cells were cultivated in phenol red free endothelial cell growth medium (ECGM) (Customer formula-

<table>
<thead>
<tr>
<th>Table 1. Substances used for stimulation of PR-A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substance</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Progesterone, nmol/ml</td>
</tr>
<tr>
<td>Mifepristone, nmol/ml</td>
</tr>
</tbody>
</table>

**Immunocytochemistry**

Expression of PR-A was analyzed by using a specific monoclonal antibody (1A6, Mouse IgG1, 1:50, Immunotech, Prague, Czech Republic) and the ABC staining method (Vectastain Elite mouse-IgG-Kit, Vector, Burlingame, Calif., USA). Staining intensity was graded by using a semiquantitative score by counting the absolute percentage of positive-stained cells.

Two blinded, independent observers evaluated the specific immunocytochemical staining reaction without knowing the prior evaluation of each specimen. In each condition, 6–9 independent specimens were taken and evaluated independently.

In brief, HUVECs were cultivated under sterile conditions in chamber slide cultures Quadriperm (Nunc) for up to 72 h, dried, wrapped and stored at −80°C as described earlier [25]. After thawing, cells were briefly fixed with formalin (Merck; 5% in PBS, 5 min). Slides were incubated in methanol/H₂O₂ (30 min) to inhibit endogenous peroxidase activity, washed in PBS (5 min) and treated with goat serum (20 min, room temperature, RT) to reduce nonspecific background staining. Incubation with the primary anti-PR-A antibody (1A6, Mouse IgG1, 1:50, Immunotech) was done overnight at 4°C. Sections were then incubated with the biotinylated secondary anti-mouse antibody (1 h, RT) and avidin-biotinylated peroxidase (45 min, RT). Between each step, the sections were washed with PBS (pH 7.4), three times. Peroxidase staining reaction was done with diaminobenzidine/H₂O₂ (1 mg/ml; 5 min) and stopped in tap water (10 min). Sections were counterstained in hematoxylin (1 min) and then coverslipped. In controls, the primary antibody was replaced with preimmune mouse serum. Positive (MCF-7 breast cancer cell line) and negative control cells (MDA-MB231), both from American Type Culture Collection (Manassas, Va., USA), for PR-A staining were always included. The slides were finally
Effects of Progesterone and Its Antagonist on HUVECs

**Results**

**Progesterone**

Progesterone acts as a natural PR ligand (PR-A, PR-B). HUVECs stimulated with 100 pmol/ml and 1, 10, and 100 nmol/ml progesterone, respectively, showed expression of PR-A after cultivation for up to 72 h (fig. 1a–e). Significantly elevated PR-A expression could be observed by administration of 10 and 100 nmol/ml progesterone, as described (p < 0.05; fig. 1f).

**Mifepristone**

Unexpectedly, administration of the PR antagonist RU486 led to an upregulation of PR-A expression. HUVECs stimulated with 1, 10, and 100 nmol/ml mifepristone showed significant upregulation of PR-A in all cases compared with nonstimulated controls (p < 0.05, respectively; fig. 2).

**Discussion**

Recently, basic findings on the expression of estrogen receptor (ER) and PR in HUVECs were published, indicating the lack of ERα and PR-B expression in HUVECs [25].

In our study, we were able to demonstrate that progesterone acts as an activator on endothelial cells and is able...
upregulate PR-A expression in a dose-dependent manner. In contrast to former studies, PR antagonist RU486 also led to an upregulation of PR-A expression.

The cellular effects of gestagens are mediated by binding to nuclear receptors (PR) which activate transcription of genes involved in cellular growth control. So far, endothelial effects of gestagens are poorly understood and, as compared with data on estrogenic influences on endothelial cells, also poorly investigated. Fu et al. [26] investigated effects of progesterone and MPA on actin remodeling, moesin activation and cell movement in human endothelial cells. They were able to show that both gestagens regulate endothelial cell movement by rapidly signaling to the actin-binding protein moesin and to the actin cytoskeleton.

To further study the effects of gestagens on vascular function, Hermenegildo et al. [27] studied the effects of progesterone and MPA on prostacyclin production in HUVECs. Both gestagens significantly increased prostacyclin release in a time- and dose-dependent manner by enhancing Cox-1 and Cox-2 expression and activities.

In contrast to our recent findings on the lack of PR-B expression on HUVECs, Tatsumi et al. [28] described PR-A and PR-B mRNA expression on HUVECs. The authors investigated the effect of progesterone, MPA, norethindrone acetate, levonorgestrel as well as dienogest on cytokine-stimulated HUVEC expression of adhesion molecules. However, progesterone or dienogest did not affect IL-1β-stimulated ICAM-1 or VCAM-1 expression, whereas the other gestagens did.

Additionally, concomitant addition of mifepristone blocked the gestagen-induced increase in adhesion molecules. They concluded that dienogest unlike other synthetic progestins lacks the stimulatory effect on cell adhesion molecules [28]. We were able to show that HUVEC stimulation with increasing amounts of RU486 leads to an upregulation of PR-A in a dose-dependent manner. Our data implicate that RU486 only acts as a PR antagonist in the presence of PR activators like progesterone.

The Women’s Health Initiative trial reported an excess of heart diseases in postmenopausal women receiving MPA. Therefore, Simoncini et al. [21] investigated the effects of progesterone, MPA, dydrogesterone and its me-

---

Fig. 2. a PR-A expression in unstimulated HUVECs. ×10. b PR-A expression in HUVECs after 0.1 nmol/ml stimulation with mifepristone. ×10. c PR-A expression in HUVECs after 1.0 nmol/ml stimulation with mifepristone. ×10. d PR-A expression in HUVECs after 10 nmol/ml stimulation with mifepristone. ×10. e PR-A expression in HUVECs after 100 nmol/ml stimulation with mifepristone. ×10. f PR-A expression in HUVECs after incubation with mifepristone.
tabolite 20-a-dihydrogesterone on endothelial synthesis of NO, and characterized the signaling events recruited by these compounds. In contrast to dydrogesterone, progestosterone and 20-a-dihydrogesterone, MPA did not trigger eNOS enzymatic activation and decreased the extent of eNOS induction by estradiol. The authors concluded that their findings support the concept that synthetic progestins act differently on vascular cells and that hormonal preparations may differ in their cardiovascular effects [29].

Studies on the effects of HT in postmenopausal women indicate procoagulant effects of gestagens and estrogens. To further study the effects of gestagens on hemo-
stasis, Zerr-Fouineau et al. [30] investigated whether pro-
gestins affect the formation of NO in endothelial cells and examined the underlying mechanism.

Certain progestins, including MPA, reduced the anti-
aggregatory effect of endothelial cells by decreasing the expression of eNOS and the formation of NO in endothelial cells; an effect that is mediated via activation of glu-
corticoid receptors.

In summary, our study showed that progesterone is able to upregulate PR-A expression in a dose-dependent manner and that the PR antagonist mifepristone also acts as a PR activator when administered solely.

Acknowledgements

This study is part of the doctoral thesis of Gitti Saadat and A-
run Geller. Bettina Toth was supported by ‘Friedrich Baur-Stif-
tung’, ‘Förderung für Forschung und Lehre’, ‘Hochschul-Wissen-
chafts-Programm’ and LMU Excellent Mentoring Program, Ludwig Maximilians University, Munich, Germany.

References

1 Feletou M, Vanhoutte PM: Endothelial dys-
function: a multifaceted disorder (The Wig-
gers Award Lecture). Am J Physiol Heart
2 Taddei S, Virdis A, Ghidoni L, Mattei P, Su-
dano I, Bernini G, Pinto S, Salvetti A: Meno-
pause is associated with endothelial dys-
fuction in women. Hypertension 1996;28:
576–582.
3 Herrington D: Role of estrogens, selective es-
trogen receptor modulators and phytoestro-
gens in cardiovascular protection. Can J
Cardiol 2000;16(suppl E):5E–9E.
4 Kublickiene K, Svedas E, Landgren BM,
Crisby M, Nahar N, Nisell H, Poston L: Small
artery endothelial dysfunction in postmeno-
pausal women: in vitro function, morphol-
ysis, and modification by estrogen and selec-
tive estrogen receptor modulators. J Clin
Endocrinol Metab 2005;90:6113–6122.
5 Majmudar NG, Robson SC, Ford GA: Effects
of the menopause, gender, and estrogen re-
placement therapy on vascular nitric oxide
activity. J Clin Endocrinol Metab 2000;85:
1577–1583.
6 Kannel WB: The Framingham study. BMJ
7 Low AK, Russell LD, Holman HE, Shepherd
JM, Hicks GS, Brown CA: Hormone replace-
ment therapy and coronary heart disease in
8 Gouva L, Tsatsoulis A: The role of estrogens
in cardiovascular disease in the aftermath of
clinical trials. Hormones (Athens) 2004;3:
171–183.

9 Rosano GM, Vitale C, Fini M: Hormone re-
placement therapy and cardioprotection: what is good and what is bad for the cardio-
vascular system? Ann NY Acad Sci 2006;
10 Rossouw JE, Anderson GL, Prentice RL, La-
Croix AZ, Kooperberg C, Stefanick ML,
Jackson RD, Beresford SA, Howard CV,
Johnson KC, Kotchen JM, Ockene J: Risks
and benefits of estrogen plus progestin in
healthy postmenopausal women: principal
results From the Women’s Health Initiative
randomized controlled trial. JAMA 2002;
11 Lemay A: The relevance of the Women’s
Health Initiative results on combined hor-
mone replacement therapy in clinical prac-
715.
12 Barrett-Connor E, Bush TL: Estrogen and
coronary heart disease in women. JAMA
13 Manson JE, Hsia J, Johnson KC, Rossouw JE,
Assaf AR, Lasser NL, Trevisan M, Black HR,
Heckbert SR, Detrano R, Strickland OL,
Wong ND, Crouse JR, Stein E, Cushman M:
Estrogen plus progestin and the risk of coro-
nary heart disease. N Engl J Med 2003;349:
523–534.
14 Tannen RL, Weiner MG, Xie D, Barnhart K:
A simulation using data from a primary care
practice database closely replicated the wom-
’en’s health initiative trial. J Clin Epidemiol
2007;60:686–695.
15 Sharma S: Hormone replacement therapy in
menopause: current concerns and consider-
293.
16 Tansavatdi K, McClain B, Herrington DM:
The effects of smoking on estradiol metabo-
17 Bagchi D, Das DK, Tosaki A, Bagchi M, Ko-
thari SC: Benefits of resveratrol in women’s
248.
18 Dhiman RK, Chawla YK: Is there a link be-
tween oestrogen therapy and gallbladder
129.
19 Hsia J, Langer RD, Manson JE, Kuller L,
Johnson KC, Hendrix SL, Pettinger M, Heck-
bert SR, Greep N, Crawford S, Eaton CB,
Kostis JB, Caralis P, Prentice R: Conjugated
equine estrogens and coronary heart disease:
the Women’s Health Initiative. Arch Intern
20 Vickers MR, Martin J, Meade TW: The
Women’s international study of long-dura-
tion oestrogen after menopause (WISDOM):
a randomised controlled trial. BMC Wom-
ens Health 2007;7:2.
21 Simoncini T, Mannella P, Fornari L, Caruso
A, Willis MY, Garibaldi S, Baldacci C, Genazzani AR: Differential signal transduc-
tion of progesterone and medroxyprogeste-
one acetate in human endothelial cells. En-
22 Adams MR, Clarkson TB, Shively CA, Parks
JS, Kaplan JR: Oral contraceptives, lipopro-
teins, and atherosclerosis. Am J Obstet Gy-


