Sex Steroids Used in Hormonal Treatment Increase Vascular Procoagulant Activity by Inducing Thrombin Receptor (PAR-1) Expression
Role of the Glucocorticoid Receptor

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Background—The use of sex steroids in oral contraception or hormonal replacement therapy is associated with an increased risk of cardiovascular thromboembolic complications. Although both the estrogen and the progestin components have been involved, the underlying mechanisms responsible are unclear.

Methods and Results—This study examined whether sex steroids promote hemostasis indirectly by increasing the procoagulant activity of blood vessels. Treatment of vascular smooth muscle cells with several progestins (progesterone, 3-keto-desogestrel, gestodene, and medroxyprogesterone acetate) upregulated proteolytically activatable thrombin receptor (PAR-1) expression, resulting in a potentiated thrombin-induced tissue factor expression and surface procoagulant activity. In contrast, neither the progestins levonorgestrel, norethisterone, and norgestimate nor the synthetic estrogen 17α-ethinylestradiol had such effects. The effect of the stimulatory progestins, which induce glucocorticoid-like effects in several cell systems, was mimicked by dexamethasone and inhibited by the progesterone and glucocorticoid receptor antagonist RU-38486. In addition, long-term administration of progesterone, 3-keto-desogestrel, or medroxyprogesterone acetate to ovariectomized rats increased PAR-1 protein level in the arterial wall, resulting in an increased responsiveness of isolated aortic rings to thrombin.

Conclusions—These data demonstrate that several progestins markedly potentiate the vascular procoagulant effects of thrombin by increasing the availability of membrane thrombin receptors in the smooth muscle, an effect that is most likely due to their glucocorticoid-like activity. (Circulation. 2001;104:2826-2831.)

Key Words: thrombin tissue factor thrombosis muscle, smooth progestin

Numerous clinical trials indicate that estrogen-progestin combinations used for oral contraception or postmenopausal replacement therapy increase the relative risk of venous thromboembolic events (VTEs) 2- to 4-fold. These complications can be observed particularly in first-time users and during the first year of treatment, and they increase further in women with risk factors for venous thromboembolism, such as obesity. The higher incidence of VTEs was attributed initially to the estrogen component of oral contraceptives (OCs), because the reduction from 50 to 30 μg of estrogen (low-dose estrogen in newer preparations) per tablet had a favorable outcome. Several recent independent studies, however, have shown a 2-fold increased relative risk of VTEs with combined OCs containing either gestodene or desogestrel in combination with low-dose estrogen compared with those containing levonorgestrel and similar low-dose estrogen. Although a certain selection bias cannot be entirely excluded, these findings highlight a potential determinant role of the progestin component of OCs for the development of VTEs.

Thrombotic events are the result of complex local processes involving not only humoral factors of the hemostatic and fibrinolytic systems but also blood constituents (eg, platelets) and cells from the vascular wall. Progestins appear to have negligible influence on the moderate increase in coagulation and fibrinolytic activity in plasma induced by estrogens in women. The recent observation of a reversible acquired resistance to activated protein C during intake of OCs, which was more pronounced with formulations containing gestodene and desogestrel than levonorgestrel, might help to explain the increased risk of VTEs.

In the present study, we examined whether sex steroids induce vascular tissue factor (TF)–dependent procoagulant activity by either a direct or an indirect effect by potentiating
the response to thrombin. The investigations were performed with vascular smooth muscle cells (VSMCs), because they are a major cellular source of TF, the physiological trigger of the blood clotting system, in blood vessels.

Methods

Cell Culture
VSMCs were isolated from Wistar rat aorta and cultured as described. Human aortic SMCs were obtained from Clonetics and cultured as recommended. Confluent cells were serum-deprived for 24 hours before experiment.

Northern and Western Blot Analyses
PAR-1 mRNA and TF mRNA levels were assessed by Northern blot analysis as described. The PAR-1 cDNA probe was a 1400-bp-long EcoRI restriction fragment from a Chinese hamster PAR-1 cDNA clone (provided by Dr Van Obberghen-Schilling, Nice, France). The TF cDNA probe was an 1100-bp-long PstI restriction fragment from a mouse TF cDNA clone (provided by Dr Nawroth, Tübingen, Germany). Phenol-soluble proteins from VSMCs or homogenized rat aortic rings were separated by SDS-PAGE (8%) and transferred to nitrocellulose membranes. The immunoreactive band was detected by enhanced chemiluminescence (Amersham).

Animal Treatment and Organ Chamber Studies
Wistar rats (16 weeks old) were ovariectomized, followed by a 2-week rest period. They were treated subcutaneously with either estrogen 17\(\beta\)-ethinylestradiol (EE) or a progestin (progesterone 16.7 mg/kg, 3-keto-desogestrel [KDG] 0.5 mg/kg, or medroxyprogesterone acetate [MPA] 16.7 mg/kg) every day for 7 days. The aorta was excised and cut into rings, which were either suspended in organ chambers with vascular smooth muscle cells (VSMCs) were determined with the chromogenic substrate S-2238 (Hemochrom Diagnostica). Optical densities were measured in a spectrophotometer at 405 nm.

Statistical Analysis
All values are reported as mean±SEM. Statistical analysis was performed with Student’s \(t\) test or ANOVA followed by Student-Newman-Keuls \(t\) test where appropriate. A value of \(P<0.05\) was considered statistically significant.

Results

Sex Steroids Induce Thrombin Receptor Expression
The progestin KDG, the active metabolite of desogestrel, significantly increased PAR-1 mRNA levels in VSMCs in a time-dependent manner (Figure 1A). PAR-1 mRNA levels were also significantly increased by the progestins progesterone, gestodene, and MPA, whereas the progestins levonorgestrel, norgestimate, and norethisterone and the synthetic estrogen 17\(\alpha\)-ethinylestradiol had only minor effects (Figure 1B). The stimulatory effect of KDG and MPA on PAR-1 at the mRNA level was observed at concentrations of \(\geq10\) nmol/L (Figure 1, C and D). To determine PAR-1 expression at the protein level, Western blot analysis was performed with a monoclonal antibody directed against the peptide sequence GRAVYLNKSRFPMPPPFPFISEDAGS in the N-terminus below the thrombin cleavage site of the rat PAR-1 (COR7-6H9, provided by Dr Ramakrishnan, COR Therapeutics). In VSMCs, a faint band of \(\approx70\) kDa was detected, which appears to be specific for PAR-1, because it could be eliminated by competition with a 10-fold molar excess of the synthetic immunizing peptide (data not shown). Exposure of VSMCs to MPA for 24 hours increased the intensity of this 70-kDa band, indicating an upregulation of membrane thrombin receptors (Figure 1E). Importantly, the stimulatory effect of progestins is observed at concentrations within the therapeutic range. Peak serum concentrations of KDG in women taking the combination of 30 \(\mu\)g ethinylestradiol and 150 \(\mu\)g desogestrel were \(\approx10\) nmol/L, those of gestodene were \(\approx30\)
nmol/L after intake of the combination of 30 μg ethinylestradiol and 75 μg gestodene,14 and those of MPA were 5 nmol/L after intake of 2 mg 17β-estradiol and 5 mg MPA.15

Role of the Glucocorticoid Receptor
The stimulatory progestins (KDG, progesterone, gestodene, and MPA) have been shown to bind not only to the progesterone but also to the glucocorticoid receptor with relatively high affinity and to induce glucocorticoid-like effects in several experimental cell systems. In contrast, the inactive progestins (levonorgestrel, norethisterone, norgestimate) bind almost exclusively to the progesterone receptor and do not bind to the glucocorticoid receptor.16,17 Therefore, we analyzed the role of the glucocorticoid receptor in regulating PAR-1 expression. The glucocorticoid dexamethasone upregulated PAR-1 mRNA and protein levels with a threshold concentration of 1 nmol/L (Figure 2, A and B). The stimulatory effect of progestins and dexamethasone was abolished by RU-38486, a potent progesterone and glucocorticoid receptor antagonist (Figure 2C). Thus, activation of the glucocorticoid rather than the progesterone receptor appears to mediate the stimulatory effect of progestins on PAR-1 expression. The finding that RU-38486 alone slightly induced PAR-1 mRNA expression (Figure 2C) can be explained by the fact that this drug at concentrations >0.1 μmol/L can act as a partial glucocorticoid agonist.16,18

Progestins Potentiate Vascular Procoagulant Activity
Thrombin induces the expression of TF, the physiological trigger of the blood clotting system, in VSMCs.19 Therefore, we examined the possibility that progestins potentiate the thrombin-induced TF-dependent procoagulant activity. A low concentration of thrombin (0.1 U/mL, 1 hour) induced a modest signal for TF mRNA, which was markedly increased by preincubation of VSMCs with either MPA, KDG, or dexamethasone at concentrations as low as 1 nmol/L for 24 hours (Figure 3, A, B, and C). In contrast, steroids alone did not induce TF mRNA expression (Figure 3A and data not shown). To determine whether increased TF mRNA levels are associated with cell surface procoagulant activity, human VSMCs were exposed to thrombin for 6 hours, a time period that induces maximal TF activity on the surface of VSMCs.20 As expected, thrombin slightly increased the low basal procoagulant activity (significant when the data shown in Figure 4, A and B, were combined), and this response was markedly potentiated by pretreatment of VSMCs with MPA.

Figure 2. Glucocorticoid receptor activation induces PAR-1 mRNA and protein expression in VSMCs. A, Dexmethasone (DEX, 24 hours) concentration-dependently upregulates PAR-1 mRNA expression. Panels show PAR-1 mRNA, corresponding 18S ribosomal RNA, and cumulative data (n=4). B, Western blot analysis showing upregulation of PAR-1 protein level by DEX (0.1 μmol/L, 24 hours). Similar observations were made in 2 additional experiments. C, Glucocorticoid and progesterone receptor antagonist RU-38486 (RU, 10 μmol/L) prevents upregulation of PAR-1 mRNA in response to KDG, MPA, and DEX (24 hours, n=5). Cumulative data represent mean±SEM. *P<0.05 vs control (CTL); #P<0.05 vs respective progestin or DEX treatment alone.

Figure 3. Steroids and thrombin (Thr)-induced expression of TF mRNA in VSMCs. Pretreatment of VSMCs with either MPA (A), KDG (B), or dexamethasone (DEX) (C) for 24 hours concentration-dependently potentiates thrombin (0.1 U/mL, 1 hour)-induced expression of TF mRNA. Panels show TF mRNA, corresponding 18S ribosomal RNA (ethidium bromide staining), and cumulative data as mean±SEM (n=4). B and C, Similar observations were made in 2 additional experiments. *P<0.05 vs thrombin alone.
or dexamethasone for 24 hours before the addition of thrombin (Figure 4, A and B). Exposure of VSMCs to MPA or dexamethasone alone did not induce procoagulant activity (Figure 4, A and B). The thrombin-induced surface procoagulant activity of VSMCs is dependent on the availability of membrane-bound TF, because this effect was abolished by a neutralizing antibody directed against TF (Figure 4, A and B). It also involves PAR-1 activation, because an increased procoagulant activity was obtained with the combination of MPA and the direct activator of PAR-1 SFLLRNP, whereas the increased procoagulant activity was abolished by a neutralizing antibody directed against human PAR-1 (Figure 5, A and B). To examine PAR-1 expression in human VSMCs, Western blot analysis was performed with a monoclonal antibody directed against human PAR-1. A faint band of ~70 kDa was observed in control VSMCs, and the intensity of this band was markedly increased after exposure of VSMCs to either MPA or dexamethasone for 24 hours (Figure 4, A and B). Altogether, the findings indicate that several sex steroids potentiate the thrombin-induced TF-dependent surface procoagulant activity of VSMCs by increasing the number of membrane-associated PAR-1s.

In Vivo Treatment With Progestins and Vascular Reactivity

To determine whether long-term administration of progestins potentiates the vascular responsiveness to thrombin, ovariec-
tomized rats were treated with either vehicle (control group) or a progestin (progesterone, KDG, MPA), and the vascular reactivity of the isolated aorta was assessed in an organ chamber. Thrombin (10 U/mL) induced modest but consistent contractions of aortic rings without endothelium, which were significantly increased in the group of rats treated with either KDG or MPA but not progesterone (Figure 6A). In contrast to thrombin, concentration-contraction curves to phenylephrine were similar in the different groups of rats (EC50 values were 30.9±6.2, 44.9±5.5, 18.0±3.0, and 27.5±2.9 nmol/L in control and progesterone-, KDG-, and MPA-treated rats). In addition, the endothelium-dependent and nitric oxide (NO)–mediated relaxations to either thrombin or acetylcholine were similar in the control and sex steroid–treated groups of rats (data not shown). To examine PAR-1 protein levels in the aorta of each group of rats, Western blot analysis with MabCOR7-6H9 was performed. In the control group, a band of ~70 kDa was detected, which was not observed in the

Figure 4. Steroids and thrombin (Thr)-induced surface procoagulant activity of human VSMCs. A and B, Pretreatment of VSMCs with either MPA (0.1 μmol/L) or dexamethasone (DEX) (0.1 μmol/L) for 24 hours potentiates thrombin (1 U/mL, 6 hours)–induced surface procoagulant activity determined in recalcified platelet-poor plasma. Inhibitory effect of neutralizing antibody (Ab) directed against human TF is also shown (Mab 4508, 10 μg/mL; American Diagnostica). Data represent mean±SEM (n=6 to 8). Insets show representative Western blot analyses indicating that MPA and DEX (0.1 μmol/L, 24 hours) upregulate PAR-1 protein level in VSMCs. Similar observations were made in 2 additional experiments. *P<0.05 vs thrombin alone.

Figure 5. Steroids and PAR-1–mediated surface procoagulant activity of human VSMCs. A, Pretreatment of VSMCs with MPA (0.1 μmol/L, 24 hours) potentiates thrombin receptor–activating peptide (TRAP) (100 μmol/L, 6 hours)–induced surface procoagulant activity. Data represent mean±SEM (n=3). *P<0.05 vs TRAP alone. B, Representative experiment showing that a neutralizing antibody directed against human PAR-1 (MabN77-300M, 50 μg/mL; Biodesign) abolished surface procoagulant activity induced by exposure of VSMCs to dexamethasone (DEX) for 24 hours followed by thrombin (1 U/mL, 6 hours). Similar observations were made in 2 additional experiments.
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 in target cells. Surprisingly, upregulation of vascular PAR-1 expression occurred selectively with certain progestins, such as progesterone, gestodene, KDG, and MPA, whereas levonorgestrel, norgestimate, and norethisterone, despite their similarly strong progestin potency, and also the estrogen 17α-ethinylestradiol did not. The effect of the stimulatory steroid hormones was inhibited by RU-38486, suggesting the involvement of the progesterone and/or glucocorticoid receptor. Because all stimulatory progestins have distinct intrinsic glucocorticoid activity as opposed to the inactive ones, and the glucocorticoid dexamethasone is a potent inducer of vascular smooth muscle PAR-1 expression, activation of the glucocorticoid receptor rather than of the progesterone receptor appears to be a key event in the steroid-induced upregulation of PAR-1 expression. Moreover, the kinetics of PAR-1 expression suggests that progestins control PAR-1 expression by an indirect mechanism possibly involving the generation of secondary regulatory molecules. Among potential candidates are growth factors, such as platelet-derived growth factor, transforming growth factor, and basic fibroblast growth factor, all of which are generated by the vascular smooth muscle and upregulate PAR-1 expression in VSMCs.

The present results also indicate that long-term administration of progestins with partial glucocorticoid activity (progesterone, MPA, and KDG) to ovariectomized rats causes a marked increase of the PAR-1 level in the aorta. The increased availability of membrane PAR-1 subsequently enhances the contraction of the vascular smooth muscle to thrombin. Surprisingly, endothelium-dependent and NO–mediated relaxations to thrombin are unaffected by the progestin treatment. Similarly, increased PAR-1 levels in the aorta of rats after long-term administration of angiotensin II correlate with an increased vasoconstriction to thrombin, whereas the endothelial formation of NO is unaltered. Thus, PAR-1 function is likely to be controlled by different mechanisms in VSMCs and in endothelial cells. Consistent with such an idea, the responsiveness of endothelial cells to thrombin appears to be controlled largely by an endogenous pool of preformed thrombin receptors, whereas in VSMCs it is controlled predominantly by de novo synthesis of the receptor. Alternatively, PAR-1 expression in endothelial cells may be controlled by endogenous regulatory factors, such as NO and prostacyclin, which have been shown to regulate the expression of several proatherosclerotic and prothrombotic genes, including those encoding adhesion molecules and TF.

The stimulatory effect of progestins on vascular PAR-1 expression both in vitro and in vivo is observed at concentrations similar to those found in plasma of women during hormonal treatment. Therefore, the present findings are likely to be of clinical significance. They suggest that an increased sensitivity of the vascular smooth muscle for expression of procoagulant activity to thrombin by functional upregulation of thrombin receptors (PAR-1) may be a key event in promoting initiation and progression of VTEs in women using OCS. In particular, such a sequence of events might provide an explanation for the 2-fold increased relative

Discussion

Thrombin is a key enzyme of the coagulation cascade and also a potent inducer of platelet activation and of procoagulant and proinflammatory responses in vascular cells. All of these events play a central role in the initiation and development of cardiovascular pathologies associated with thrombosis, such as stroke and myocardial infarction. Thrombin exerts most of its action on target cells via an irreversible proteolytic activation of PAR-1. Cellular responses to thrombin rapidly desensitize despite the irreversible mechanism of activation of PAR-1. A major consequence of this unusual mechanism of activation and desensitization of PAR-1 is that the responsiveness of cells to thrombin is critically dependent on the constant supply of new receptor molecules to the plasma membrane.

The present findings indicate that certain progestins substantially upregulate PAR-1 expression in VSMCs, resulting in the subsequent increased availability of membrane receptors. As an important consequence of these progestin-induced changes, the vascular smooth muscle becomes more responsive to thrombin in its TF-dependent procoagulant activity.
risk of VTEs and fatal pulmonary embolism in women taking combined low-dose OCs containing gestodene or desogestrel compared with those taking levonorgestrel and estrogen at a similar low dose.\textsuperscript{5} Recently, in vitro studies have shown that TF expression in endothelial cells is depressed by the endogenous formation of NO.\textsuperscript{28} Therefore, it is likely that the potentiating effect of progestins on the TF-dependent vascular procoagulant activity to thrombin may be greater in vascular pathologies characterized by a reduced bioavailability of endothelial NO, such as obesity, hypertension, and hypercholesterolemia, and possibly also in blood vessels exposed to low levels of shear stress. Shear stress is the most important physiological stimulus for the continuous formation of NO in endothelial cells. Consistent with such a concept, the relative risk of VTEs in women during hormonal treatment increases with the presence of such risk factors as high body mass index,\textsuperscript{2,3} and thrombotic events in OC users occur predominantly in the venous circulation.

An additional important finding of the present study is that, like progestins with partial glucocorticoid activity, the pure glucocorticoid dexamethasone also markedly potentiated TF-dependent procoagulant activity to thrombin by increasing the availability of membrane PAR-1 in human VSMCs. The stimulatory effect of the glucocorticoid, which does not bind to the progesterone receptor,\textsuperscript{29} is obtained at low concentrations (in the nanomolar range), suggesting that it may be of clinical relevance. Such a sequence of events may contribute to explain the increased risk of thromboembolism in patients under long-term glucocorticoid therapy or with an endogenous hypercortisolism (Cushing’s syndrome).\textsuperscript{30}

In conclusion, the present findings demonstrate that certain progestins currently used in oral contraception and in hormone replacement therapy can remarkably sensitize the vascular smooth muscle for expression of procoagulant activity to thrombin by upregulating the availability of membrane thrombin receptors (PAR-1). They further suggest that the partial glucocorticoid activity of progestins is likely to be responsible for this action and thus might be a key determinant in the induction of a thrombotic state.

References

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_Circulation_. 2001;104:2826-2831
doi: 10.1161/hc4801.099737

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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