Progestogens reduce thromboxane production by cultured human endothelial cells


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ABSTRACT

Objectives Progestogens have been poorly studied concerning their roles in endothelial physiology. Prostanoids are vasoactive compounds, such as thromboxane A₂, a potent vasoconstrictr, and prostacyclin, a vasodilator. We examined the effects of two progestogens used clinically, progesterone and medroxyprogesterone acetate, on thromboxane A₂ production by cultured human umbilical vein endothelial cells (HUVEC) and investigated the role of progesterone receptors and the enzymes involved in production of thromboxane A₂ and prostacyclin.

Methods Cells were exposed to 1–100 nmol/l of either progesterone or medroxyprogesterone acetate, and thromboxane A₂ production was measured in culture medium by enzyme immunoassay. Gene expression of prostacyclin synthase and thromboxane synthase was analyzed by quantitative real-time polymerase chain reaction. Expression of prostacyclin synthase protein was analyzed by Western blot.

Results Both progestogens decreased thromboxane A₂ release after 24 h. Protein and gene expression of prostacyclin synthase were increased after exposure to both progestogens, without changes in thromboxane synthase expression. These effects induced by progestogens were mediated through progesterone receptors, since they were decreased in the presence of the progesterone receptor antagonist RU486. The cyclooxygenase-1 selective inhibitor reduced thromboxane release.

Conclusion Progesterone and medroxyprogesterone acetate decreased HUVEC thromboxane release in a progesterone receptor-dependent manner, without changes in thromboxane synthase expression and enhanced prostacyclin synthase gene and protein expression.

INTRODUCTION

The term ‘progestogen’ designates both natural and synthetic progestational molecules, including natural progesterone, or exclusively synthetic ones such as the 17α-hydroxyprogesterone derivative medroxyprogesterone acetate (MPA), which are currently used as a second component of hormone replacement therapy in postmenopausal women with a uterus, in order to reduce the risk of endometrial cancer. The cardiovascular system is a target for progestogens, but several aspects still remain unexplained by the fact that there is controversy regarding the effects of progestogens on the vasculature.

Blood vessels and vascular cells contain functional progesterone receptors (PR). Thus, two types of PR have been described, PR-A (94 kDa) and PR-B (116 kDa), which are functionally regulated by both kinds of progestogens. Progestogens inhibit vasorelaxation, and it has been previously demonstrated that, in the absence of progesterone, loss of the PR in PR knockout mice leads to a more pronounced vascular injury, whereas, in animals with intact PR, progesterone increases the vascular injury response. On the contrary, progesterone induces endothelium-dependent attenuation of contractile responses to phenylephrine in mesenteric arterial rings through nitric oxide, or potentiates them in combination with estradiol in isolated human endothelial cells.

Vascular endothelium plays a leading role in vascular physiology, modulating vessel tone and controlling platelet adhesion and aggregation, actions mainly mediated through
the release of vasorelaxing factors such as prostacyclin (PGI2) and nitric oxide, or vasoconstrictors such as endothelin-1, angiotensin II or thromboxane A2 (TXA2).

PGI2 and TXA2 are unstable lipid mediators and, after intracellular biosynthesis, are released outside the cell and work mostly locally in an autocrine or paracrine fashion. In activated platelets, TXA2 is produced by the enzyme thromboxane synthetase (TXAS). This eicosanoid not only causes platelet aggregation, but it is also a potent vasoconstrictor and is related to the pathophysiology of acute coronary ischemic syndromes13. PGI2 is produced by prostacyclin synthase (PGIS) in vascular endothelial cells. PGI2 not only prevents platelet action and clumping, but it is also a powerful vasodilator. Both eicosanoids are synthesized from a precursor, prostaglandin PGH2, which is made by two isoforms of the enzyme cyclo-oxygenase (COX), COX-1 and COX-214–17.

We have previously shown that progesterone and MPA are able to increase PGI2 production in a PR-dependent manner where enhanced expression and activity of both COX isoenzymes were involved in its production3. Our current aims were (1) to study the effects of progesterone and MPA on TXA2 production by endothelial cells, (2) to discover whether these actions are mediated by PR, and (3) to evaluate the enzymatic pathway responsible for its synthesis.

**METHODS**

**Cell culture and experimental design**

Primary human umbilical vein endothelial cells (HUVEC) were isolated, identified and cultured as previously described3. Cells from passages 4–6 were seeded onto six-well plates with fibronectin-treated coverslips for immunocytochemistry, onto 24-well plates for PGI2 and TXA2 measurements, onto 96-well plates for cell viability measurement, and into 25 cm² flasks for Western blot and mRNA isolation. When cells were at 75% of confluence, the culture medium was exchanged for a phenol red-free medium 199 (GIBCO-BRL, Life Technologies, Paisley, UK) supplemented with 20% charcoal/dextran-treated fetal bovine serum (GIBCO-BRL) and maintained for 24 h. Then, the culture medium was eliminated and immediately replaced with phenol red-free medium 199. The desired concentrations of progesterone, MPA, and the PR antagonist, mifepristone (RU-486) (Sigma), were obtained by successive dilutions of a stock solution with ethanol and those of the selective inhibitors of COX-1 (SC-560) or COX-2 (NS-398) (Cayman Chemical, Ann Arbor, MI, USA) were obtained by successive dilutions of a stock solution with DMSO. Control cells were exposed to the same vehicles (less than 0.1% ethanol or 0.1% DMSO, respectively).

**Immunoblotting**

After 24 h of treatment with the desired products, cells were lysed and protein measured as previously described3. Equal amounts of protein (range 40–125 µg) were then separated by 10% of SDS-polyacrylamide gel electrophoresis, and the protein transferred to PVDF sheets (Biorad, Spain). Immunostaining was achieved using specific antibody anti-prostaglandin I synthase (PGIS) (cat: 100023; Cayman Chemical). Development was performed with alkaline-phosphatase-anti-rabbit secondary antibody (Sigma), followed with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (BCIP) color development reaction. Blots were digitalized using a Gelprinter PLUS (TDI, Madrid, Spain), and the densities of spots were analyzed with the program Image Gauge version 4.0 (Science Lab., 2001). Equivalent protein loading and transfer efficiency were verified by staining for β-actin (clone AC-15, catalog A5441, Sigma).

**Assay of TXA2 and PGI2**

After 24 h of treatment with the desired products, medium was collected and stored at ~20°C until thromboxane or PGI2 were measured. Culture wells were then washed with PBS and adherent cells were collected in 0.5 N NaOH for protein determination by the modified Lowry’s method using bovine serum albumin as standard18. TXA2 is rapidly hydrolyzed non-enzymatically to form the stable metabolite thromboxane B2 (TXB2), which was assessed in duplicate by a commercial enzyme immunoassay (EIA) kit (Cayman Chemical), and the results were expressed as pg thromboxane/mg protein.

The amount of PGI2 produced, calculated as the concentration of stable hydrolysis product, 6-keto-prostaglandin-F1α, was assessed in duplicate by a commercial EIA kit (Cayman Chemical). The production of PGI2 was expressed as ng PGI2/mg protein.

**RNA isolation and quantitative real-time PCR assay**

Total cellular RNA was extracted and reverse transcribed as previously reported2. For PGIS, TXAS and GAPDH (endogenous control), Taqman probes were used: Hs00168766_m1, Hs00233423_m1 and 4326317E, respectively (Applied Biosystems, Fosters City, CA, USA). Quantitative real-time polymerase chain reaction (PCR) assay was performed with TaqMan Universal Mastermix (Applied Biosystems). Reaction mix was prepared in RNase-free tubes of 0.2 ml by adding a volume of TaqMan Universal PCR MasterMix and TaqMan gene expression assay. The sample of cDNA obtained from the real-time PCR was incorporated with the necessary quantity of DEPC water to achieve a final concentration of 40 ng approximately (range 10–100 ng). The appropriate volume of each reaction mixture was transferred to a reaction plate which was then placed in a 7900HT fast real-time PCR system (Applied Biosystems) with the appropriate thermal cycling conditions (50°C/2 min, 95°C/10 min, 40 cycles; 95°C/15 s, 60°C/1 min). Each sample was amplified in triplicate for each
gene. Data were analyzed with the ABI PRISM Sequence Detection v. 1.7 analysis software (Perkin Elmer, Nieuwerkerk, the Netherlands).

Cell viability measurement

Cell viability was assessed as previously reported. None of the test compounds (progesterone, MPA, RU-486, SC-560, NS-398, and their combinations) were toxic for HUVEC (data not shown).

Statistical analysis

Values shown in the text and figures are mean ± standard error of the mean. The ANOVA test was applied for comparisons of means, and then Bonferroni’s test was performed. \( p \) Values <0.05 were considered significant. The statistical analysis was carried out using the Prism 4 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Exposure of endothelial cells to three different, physiological and near-physiological concentrations of progesterone (1–100 nmol/l) resulted in a dose-dependent decrease of TXA2 production \( (p < 0.05 \text{ vs. control values}) \), being lower with 100 nmol/l than with 1 nmol/l progesterone \( (p < 0.01) \) (Figure 1). The same concentrations of MPA also decreased TXA2 production \( (p < 0.05 \text{ vs. control values}) \), being lower at 10 nmol/l. These effects would be mediated through PR activation, since treatment of cells with the PR antagonist RU-486 (10 μmol/l) partially reversed the progesterone effect \( (p < 0.01; \text{Figure 2}) \).

To confirm the previous results from our group and others, we measured PGI2 and TXA2 production in the same culture medium. The effects afforded by progestogens on both prostanoids were completely opposite. In fact, there was increased PGI2 production with both progestogens together with decreased TXA2 production, ranging from 10 to 20% (Figure 3).

We have previously shown that both COX-1 and COX-2 protein expressions were significantly increased in HUVEC exposed either to progesterone or MPA in a PR-dependent way. Our next step was to examine COX-1 and COX-2 activities in relation to TXA2 production. For this purpose, specific COX-1 and COX-2 inhibitors were used (Figure 4). The use of 0.1 μmol/l SC-560 alone significantly decreased TXA2 production to 62% of control values \( (p < 0.01) \). When SC-560 was used in combination with progesterone or MPA, TXA2 production was reduced to the same levels obtained with SC-560 alone. No significant differences were found in comparison to TXA2 levels induced by either progestogen. NS-398 alone did not show any difference related to control values. When it was combined with either progestogen or MPA, there was a significant decrease in comparison to

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**Figure 1** Dose-dependent reduction of thromboxane B2 production by endothelial cells after exposure to progesterone or medroxyprogesterone acetate (MPA). Steroid-deprived HUVEC were exposed to different concentrations (1–100 nmol/l) of either progesterone or MPA for 24 h; the culture medium was then collected and the TXB2 concentration was measured as described in Material and Methods. Data are expressed as percentage of control values, and are mean ± standard error of the mean of four different experiments performed in duplicate. Average control values for all experiments were 767 ± 50 pg/mg protein (range: 486–1190 pg/mg protein). * \( p < 0.05 \text{ vs. control} \); ** \( p < 0.01 \text{ vs. control} \).

**Figure 2** Progesterone and medroxyprogesterone acetate (MPA) reduce thromboxane B2 production through progesterone receptor (PR) activation. Steroid-deprived HUVEC were exposed to different combinations of 10 nmol/l progesterone or 10 nmol/l MPA with 10 μmol/l RU-486 (a PR antagonist) for 24 h. Then, culture medium was collected and the TXB2 concentration was measured as described in Material and Methods. Data are expressed as percentage of control values, and are mean ± standard error of the mean of four different experiments performed in cells from different cultures in which 10–18 determinations were performed in duplicate. Average control values for all experiments were 703 ± 61 pg/mg protein (range: 226–1182 pg/mg protein). * \( p < 0.05 \text{ vs. control} \); ** \( p < 0.001 \text{ vs. control} \); † \( p < 0.01 \text{ vs. progesterone} \).
control levels, but, when the comparison was made with progestogens alone, no differences were found. Also, when a comparison was made between progesterone plus NS-398 and NS-398 alone, there was a significant decrease in TXA2 levels (Figure 4).

With the aim of studying the second-step enzymes responsible for prostanoid production, TXAS and PGIS were evaluated. TXAS mRNA expression in endothelial cells exposed to both progestogens remained unmodified after 24 h (Figure 5). On the contrary, progesterone and MPA significantly increased PGIS mRNA expression. When RU-486 was used alone or in combination with either or both progestogens, a significant decrease in mRNA expression was observed compared with either control \((p < 0.001)\) or progestogens alone \((p < 0.01)\) (Figure 6A). In a similar fashion, PGIS protein expression was significantly increased after HUVEC exposure to progesterone or MPA (Figure 6B and C). In that case, RU-486 partially reversed the effects afforded by progestogens, being indistin-

![Figure 3](image1.png)

**Figure 3** Progestogens decrease thromboxane A2 production along with increased PGI2 production. Steroid-deprived HUVEC were exposed to 10 nmol/l progesterone or 10 nmol/l medroxyprogesterone for 24 h. Then, culture medium was collected and thromboxane B2 and 6-keto-prostaglandin-F1\(\alpha\) concentration were measured as described in Material and Methods. Data are expressed as percentage of control values, and are mean \(\pm\) standard error of the mean of 12–21 duplicated determinations corresponding to four different experiments performed in cells from different cultures. *, \(p < 0.05\) vs. control; **, \(p < 0.001\) vs. control.

![Figure 4](image2.png)

**Figure 4** Role of COX-1 or COX-2 inhibition on progesterone or medroxyprogesterone acetate (MPA)-reduced thromboxane B2 (TXB2) production by human endothelial cells. Steroid-deprived HUVEC were exposed to different combinations of 10 nmol/l progesterone or 10 nmol/l MPA in the presence or absence of 0.1 \(\mu\)mol/l SC-560 (a COX-1 selective inhibitor) or 1 \(\mu\)mol/l NS-398 (a COX-2 selective inhibitor) for 24 h. Culture medium was then collected and TXB2 concentration was measured as described in Material and Methods. Data are expressed as percentage of control values, and are mean \(\pm\) standard error of the mean of five different experiments performed in cells from different cultures in which 11–33 determinations were performed in duplicate. Average control values for all experiments were 758 \(\pm\) 53 pg/mg protein (range: 306–1698 pg/mg protein). *, \(p < 0.05\) vs. control; **, \(p < 0.01\) vs. control; ***, \(p < 0.001\) vs. control; †, \(p < 0.05\) vs. NS-398.

![Figure 5](image3.png)

**Figure 5** Progesterone and medroxyprogesterone acetate (MPA) do not modify thromboxane synthetase (TXAS) mRNA expression in endothelial cells. Steroid-deprived HUVEC were exposed to different combinations of 10 nmol/l progesterone or 10 nmol/l MPA in the presence or absence of 10 \(\mu\)mol/l RU-486 (a progesterone receptor antagonist) for 24 h. Total RNA was extracted, and the relative expression of TXAS was quantified by qRT-PCR, as described in Material and Methods. Data are expressed as a percentage of control values and are mean \(\pm\) standard error of the mean of six values corresponding to six experiments.
guishable from control values. Nevertheless, they were significantly decreased in comparison with progestogens alone.

**DISCUSSION**

Our results show that progesterone and MPA are able to decrease TXA2 production by HUVEC after 24 h of incubation, through a PR-dependent mechanism, involving enhanced COX-1 activity and increased PGIS mRNA and protein expression, without changes in TXAS mRNA.

It is interesting to remark that progestogens increased at the same time as PGI2 production and decreased TXA2 production, two prostanoids with common steps at the start of synthesis and with opposite effects on vascular vessels. These results confirm previous data from our group where PGJ2 levels increased by about 50–100% of PGJ2, but contradict other studies.

There are few studies of the progestogenic effects on TXA2 levels. For instance, a reduction in TXB2 levels has been reported in postmenopausal women treated continuously with transdermal estradiol and every 2 months for 12 days with MPA, although blood was drawn in the estrogen-only phase of the cycle. Other studies have demonstrated that postmenopausal patients administered estradiol in association with norethisterone continuously had an increase of plasmatic thromboxane, possibly due to platelet activation.

The reported effects were obtained with physiological concentrations of progestogens, similar to those found in non-pregnant women. Thus, in the follicular phase, plasma progesterone levels are 0.15–0.70 ng/ml (corresponding to 0.5–2.2 nmol/l) and in the luteal phase they are 2.00–25.0 ng/ml (corresponding to 6.4–79.5 nmol/l). Also, the use of RU-486, at a dose within the adequate range to ensure PR antagonism in HUVEC, allows us to ascribe the observed effects to PR. Then, the observed effects of progestogens on PGI2 and TXA2 production and on the enzymes responsible for their synthesis would be dependent on PR activity, since they were reversed or partially reversed in the presence of RU-486.
Progestogens and thromboxane A2

PR exists as two functionally distinct isoforms, where PR-B functions as a transcriptional activator, and PR-A is transcriptionally inactive. HUVEC have been reported to express both types of PR. Then, it is possible to hypothesize that the progestogen-dependent TXA2 production by HUVEC would depend on PR-B activation, although controversy exists since a recent work reported the expression of PR-A, but not PR-B, in HUVEC.

Our results also suggest that TXA2 and PGI2 production by HUVEC would involve a genomic mechanism, since both effects were observed after 24 hour of treatment with evident changes in gene and protein expression of PGIS, and would depend on PR activation. Nevertheless, a non-genomic mechanism cannot completely be ruled out, since progesterone has demonstrated to exert vascular effects, by increasing nitric oxide synthesis via non-genomic mechanisms, which include MAPK/P3K activation.

To explain the divergent effect afforded by progestogens on PGI2 and TXA2 production, we studied the main enzymes in prostanooid synthesis, COX-1 and COX-2. COX-1 is generally believed to be ubiquitously expressed (termed as a constitutive isoform), whereas COX-2 is induced by mitogens, growth factors, bacterial endotoxin, and cytokines (inducible COX). Nevertheless, in endothelium, matters may not be so simple, and both COX enzymes shared characteristics of constitutive and inducible enzymes.

Previous data from our group have demonstrated a basal, constitutive protein expression of both COX in HUVEC that was raised after exposure to both progestogens. Our current outcomes suggest that TXA2 would depend on COX-1 activity, since SC-560 decreased it by 40% and NS-398 did not affect TXA2 production. Although both PGI2 and TXA2 are generated from the same unstable prostaglandin H2 produced by COX, it has been proposed, in cultured cells, that PGIS preferentially couples with COX-2 for the production of PGI2, whereas COX-1 couples with TXS.

In spite of the fact that SC-560 only partially inhibited TXA2 production, the residual TXA2 not inhibited by both COX inhibitors could reflect the release of intracellular TXA2 produced before the exposure to inhibitors, or the existence of other sources of thromboxanes. In fact, the use of indomethacin, a non-selective COX inhibitor, in the same experimental model was not able to completely inhibit prostanooid release, retaining a residual production of PGI2.

Vascular wall plays a leading role in the regulation of tone in the normal female vasculature, as a major source of constrictor prostanooids (TXA2 and PGH2), and it has been shown that inhibition of the TXAS pathway and accumulation of the common upstream intermediate PGH2 enhances the production of PGI2. Furthermore, in the systemic circulation, the vasodilatory prostanooid PGI2 is the major product of the vascular wall, whereas TXA2 is produced in much lower quantities, the reason why the TXA2/PGI2 ratio is quite low. Consequently, we may propose that progestogens, in spite of not them significantly affecting

TXAS gene expression, may decrease TXA2 production as a consequence of an increased PGIS gene and protein expression triggering the PGI2 production (Figure 7), which in turn would promote a favorable vascular balance by decreasing even more the TXA2/PGI2 ratio.

Taking together our current and previous outcomes, we propose that exposure of HUVEC to progestogens would result in a PR-dependent increase in PGI2 and a decrease in TXA2 production. That effect would be due increased COX-1 and COX-2 activity, which in turn should increase the prostanooid intermediate PGH2 content. The increased PGIS expression would turn aside the PGH2 towards PGI2 increased synthesis, whereas the decreased TXA2 production is in accordance with the unaltered TXAS expression (Figure 7).

Our outcomes contribute to the understanding of the actions exerted by progestogens and hormone replacement therapy at the vascular level in postmenopausal women. Nevertheless, we have to be cautious, since clinical studies implicate progestogens as vasoconstrictors, but, on the contrary, basic studies support a direct vasorelaxant effect. The factors responsible for this discrepancy are unknown, but may involve a combination of both genomic and non-genomic effects responsible for the clinical observations, with only non-genomic effects playing a role in the rapid in vitro effects. Our results clearly show a dual role for progestogens. On the one hand, they stimulate a decrease in TXA2 production and, on the other, they increase PGI2 production, conferring them with a protective effect on the vascular endothelium, discarding previous reports in which this was denied and showing a profile that has been already described for estrogens.
In conclusion, our results support an active role for progestogens in endothelial physiology, resulting in a beneficial profile that includes decreased TXA2 production together with an increased PGI2 production. These changes are due to modifications in the expression of the enzymes responsible for prostanoid synthesis through a PR-dependent mechanism.

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References


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