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The role of sex steroids on cellular events involved in vascular disease

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ABSTRACT

In this work we checked the hypothesis whether estrone, progesterone, and testosterone are able to modulate the interactions between platelets, monocytes, and endothelial cells either under basal or inflammatory conditions. Using adhesion assays we demonstrated that pretreatment of endothelial cells with estrone, progesterone, or testosterone prevented monocytes and platelets adhesion induced by the proinflammatory agent bacterial lipopolysaccharide. The hormones reduced the expression of mRNA of ICAM-1, VCAM-1, and P-selectin, endothelial surface proteins that mediate monocytes and platelets adhesion respectively. Integrins are the main leukocyte proteins that allow firm adhesion. Using flow cytometry we showed that estrone treatment of monocytes reduced CD11b and CD11c expression, either under basal or injury (lipopolysaccharide) conditions. The three steroids inhibited platelet aggregation in a nitric oxide dependent manner. Platelet function was not affected by the steroid treatment. The molecular mechanisms of action exerted by the steroids included the participation of the intracellular signaling pathways PKC, MAPK, and PI3K, which selectively and differentially mediate the stimulation of nitric oxide release. We evidence that estrone, progesterone, and testosterone modulate monocyte and platelet adhesion to endothelial cells, events that play a major role in the initiation and progression of vascular lesions. The steroid action was evidenced under basal or inflammatory conditions. The mechanisms of action exerted by the steroids included stimulation of nitric oxide production and the participation of PKC, MAPK, and PI3K systems.

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1. Introduction

The endothelium is the main regulator of vascular physiology that contributes to maintain vascular tone and non-thrombogenic properties of the endothelial surface [1]. A major consequence of vascular dysfunction is the atherosclerosis, a chronic inflammatory and multifactorial process, characterized by the formation of atheromatous plaques, arterial wall thickening, and lumen narrowing [2].

Vascular injury results in endothelial dysfunction and initiates atherosclerosis. The early phase of plaque generation is characterized by impaired synthesis of vasodilator compounds, such as nitric oxide (NO) and prostacyclin, subsequently followed by morphological changes, enhanced leukocyte adhesion and endothelial transmigration, platelet adhesion and aggregation, and alterations in cell growth and migration [3]. Leukocyte and platelet adhesion to the endothelium depends on the enhancement in cell adhesion molecules (CAMs) expression, cell surface glycoproteins that join leukocyte/platelet with endothelial cells. In monocyte adhesion, selectins are responsible for the initial stages of cell rolling, integrins participate in firm adhesion, and immunoglobulin superfamily mediates the transmigration step. Platelets adhesion is initially mediated by selectins and later strengthened by integrins. The antiatherogenic properties of endothelium depend on its ability to produce vasoactive compounds. NO is a potent vasodilator that inhibits endothelial cells (ECs) monocyte adhesion, vascular smooth muscle cells (VSMCs) migration and proliferation, platelets aggregation, and leukocytes integrins synthesis.

Vascular function is regulated by several factors and agonists, including sex steroid hormones. The presence for 17-β-estradiol (E_2), progesterone (Pg), and testosterone (T) receptors in ECs and VSMCs have been widely demonstrated [4]. The menopausal transition involves a progressive decline in ovarian steroidogenesis. Although beyond menopause, the plasmatic levels of E_2 markedly drop, the ovarian synthesis of androgens, progestins, and estrone (E_1) are also affected [5]. Moreover, in postmenopausal women,

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circulating levels of E_1 remain unchanged due to its synthesis in peripheral tissues through androstenedione aromatization [6]. Therefore, during menopause the contribution of sex steroids to the regulation of vascular homeostasis is altered. It has been assumed that hormone replacement therapy (HRT) possesses cardioprotective properties [7]. However, recent trials failed to show advantages in the prevention of primary or recurrent cardiovascular events in women receiving HRT with E_2 or E_2 plus Pg [8]. The reason for this fact is still unclear.

Vascular effects of E_2 have been extensively studied [7,9]. In contrast, the biochemical actions of Pg, E_1, and T on vessel wall have been scarcely investigated. Pg induces vasorelaxation [10], inhibits vascular cell adhesion molecule-1 (VCAM-1) synthesis and intercellular adhesion molecule-1 (ICAM-1) expression [11]. It has been reported that T and dehydroepiandrostenedione induce vasorelaxation due to an enhanced NO synthesis [12]. Indeed, androgens regulate cellular growth and apoptosis, and promote angiogenesis [13].

In our laboratory, we study the vascular action of E_1, Pg, and T in rat aortic tissue. The mechanism of action displayed by the steroids involves an integration of genomic and non genomic effects. The fast effects elicited by E_1 and Pg imply signal transduction activation of mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) cascades, events required for the regulation of vasoactives production, and also necessary for the modulation of ECs and VSMCs growth [14–16]. T stimulates NO synthesis and modulates endothelial growth in a NO dependent manner [17].

The aim of the present work was to evaluate whether E_1, T, or Pg was able to modulate key processes involved in the early steps of vascular diseases such as interactions between platelets, monocytes, and ECs. The hormonal actions were studied either under basal or inflammatory conditions.

2. Materials and methods

2.1. Materials

Pg was purchased from Calbiochem–Novabiochem International (San Diego, CA). Griess reagents were purchased from Britania Laboratories (Buenos Aires, Argentina). Trypsin/EDTA (10×), L-glutamine (100×), amphotericin B (0.25 mg/mL), penicillin/streptomycin (100×), and fetal calf serum (FCS) were obtained from PAA Laboratories (Pasching, Austria). RT-PCR RNA kit and Superscript III CellsDirect cDNA synthesis system were purchased from Invitrogen (Carlsbad, CA, USA). E_1, T, Dulbecco's modified eagle's medium (DMEM), lipopolysaccharides (LPS) from *Escherichia coli* 0127, N-nitro-L-arginine methyl ester (L-NAME), L-N^6-(1-iminoethyl) lysine hydrochloride (L-NIL) and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA). Matching isotype controls were purchased from BD Biosciences.

2.2. Animals

Female Wistar rats were housed under controlled conditions (constant room temperature, 12 h light/12 h dark cycle, bred in our own colony, and fed with a standard rat chow diet and free access to water). Animals aged 3–5 weeks old and 120 g of weight were killed by cervical dislocation. All the procedures were in accordance with the guidelines published in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. All animal work was performed at the Unit of Animal Care belonging to the Department of Biology, Biochemistry and Pharmacy at the University. The Animal Care Use Committee of this Unit approved the protocol used.

2.3. ECs culture

ECs cultures were obtained from rat aortic rings explants as previously described [14]. Briefly, animals were killed by cervical dislocation and the full length thoracic aorta was aseptically removed and placed in sterile and cold phosphate-buffered saline (PBS). Immediately after, the aorta was cleaned of adherent connective tissue, and cut into small ring-shaped segments. Rings were seeded in a 60 mm matrix-coated Petri dishes (NUNC) containing DMEM supplemented with 20% FCS, 60 μg/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin-B, 2 mM L-glutamine, and 1.7 mg sodium bicarbonate, and they were incubated at 37 °C in 5% CO_2 atmosphere. After 5 days of culture ring explants were removed and the remaining cells were allowed to reach confluence. The identity of the ECs was determined: (a) by phase-contrast microscope observation of the characteristic morphology of cobblestone shape growth in confluent monolayer, (b) by the positive immunocytochemistry reactivity to Factor VIII and to anti-Vimentin, clone V9 using DakoCytomation EnVision system, and (c) by the ability to synthesize NO. Cells from passages 2–7 were used for all experiments. Fresh DMEM containing 10% FCS was replaced every 72 h. Hormone solutions employed in the in vitro treatments were prepared using isopropanol as solvent. The final concentration of the vehicle was always below 0.1%. Control groups received vehicle alone.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) assay

ECs were cultured in DMEM (10% FCS) and allowed to grow to 90% of confluence. Monolayers were starved for 24 h with serum-free medium, and then treated with Pg, E_1, T or LPS (1 μg/mL). Total cellular RNA extraction and reverse transcription were performed using Superscript III CellsDirect cDNA synthesis system (Invitrogen, CA, USA) according to the instructions of manufacturer and as previously described [18]. Complementary DNA was then amplified by PCR using a programmed thermocycler (Biometra Uno II; Biometra, Göttingen, Germany). PCR cycles were as follows: P-selectin (94 °C, 3 min, 94 °C, 30 s, 62 °C, 45 s, 72 °C, 45 s, 72 °C, 7 min, 38 cycles); ICAM-1 (95 °C, 3 min, 94 °C, 60 s, 64 °C, 60 s, 72 °C, 60 s, 72 °C, 7 min, 32 cycles); VCAM-1 (95 °C, 3 min, 95 °C, 60 s, 55 °C, 60 s, 72 °C, 60 s, 72 °C, 7 min, 32 cycles). Primers sequences were as follows: P-selectin, forward: 5′-TAA TCC CCC GCA GTG TAA AG-3′, reverse: 5′-AGG TTG GCA ATG GTT CAC TC-3′; ICAM-1, forward: 5′-CTG CAG AGC ACA AAC AGC AGA G-3′, reverse: 5′-AAG GCC GCA GAG CAA AAG AAG C-3′; VCAM-1: forward: 5′-TAA GTT ACA CAG CAG TCA AAT GGA-3′, reverse: 5′-CAC ATA CAT TGC CGG AAT CTT-3′.The expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was checked for each set of RT-PCR experiments (forward primer: 5′-TCC CTC AAG ATT GTC AGC AA-3′, reverse primer: 5′-AGA TCC ACA ACG GAT ACA TT-3′; amplification steps: 95 °C, 3 min, 94 °C, 30 s, 53 °C, 30 s, 72 °C, 45 s, 72 °C, 7 min, 35 cycles). Negative controls (PCR reaction without RT product) were also processed. PCR amplification products were detected by electrophoresis in agarose gels stained with ethidium bromide. The density of each band on RT-PCR gel was quantified using Image J software (1.43 c version, NIH, Rasband). The mRNA in each sample was normalized against GAPDH mRNA.

2.5. Monocytes adhesion to ECs

2.5.1. Peripheral blood mononuclear cell and monocytes isolation

Separation of peripheral blood mononuclear cells was performed by density gradient. Heparinized whole blood diluted with PBS (1:1) was carefully layered on top of a Ficoll-Paque Plus gradient and centrifuged at 400 × g for 35 min. Mononuclear cell

interface was collected, and viability was checked with trypan blue staining. Peripheral blood mononuclear cells were suspended in DMEM supplemented with 10% FCS and placed on 35 mm Petri dishes (2×10^7 cells/mL) for 1 h at 37 °C to allow peripheral blood monocytes (PBM) adhesion. Monocytes morphology was determined by May Grünwald–Giemsa staining. The culture medium containing non-adherent cells was removed. Adhered cells were incubated for 15 min in PBS–EDTA 10 mM, washed and cultured in DMEM supplemented with 10% FCS, 60 mg/mL penicillin, and 100 mg/mL streptomycin for 72 h. Medium was replaced at 48 h. Adherent cells were detached using a scraper and suspended in DMEM. Absolute number of PBM was counted using an automatic counter. Viability was newly confirmed with trypan blue staining assay [18].

2.5.2. Monocytes adhesion assay

ECs were seeded on 12-multiwell culture plates (NUNC) at a density of 2×10^4 cells/well in DMEM supplemented with 10% FCS and allowed to grow to 90% of confluence. ECs were starved for 24 h with serum-free medium, and then exposed to 10 nM Pg, 10 nM E_1, 1 nM T or vehicle control (isopropanol <0.01%), in the presence and absence of 1 μg/mL bacterial LPS (last 21 h of hormonal treatment) in DMEM (1% FCS) as indicated in each experiment. An exact number of PBM were seeded on pretreated ECs and incubated for 2 h at 37 °C in a humidified 5% CO_2 atmosphere. Supernatants of each well containing non-adhered PBM were collected and counted. The number of adhered PBM to ECs was calculated by difference between total mononuclear seeded and non-adherent PBM. ECs and adhered PBM were dyed using Giemsa stain. Images (200×) were obtained using an OLYMPUS C7070WZ optical microscope. Results are expressed as means and standard deviations of the number of cells counted [18].

2.6. Platelet aggregation assay

Platelet aggregation was measured using a turbidimetric assay. ECs were seeded on 24-multiwell culture plates (NUNC) at a density of 3×10^4 cells/well in DMEM supplemented with 10% FCS and allowed to grow to 90% of confluence. Culture medium was replaced by 400 μL of platelet-rich plasma (PRP) (3×10^8 platelets/mL) and exposed to 10 nM Pg, 10 nM E_1 or 1 nM T for 5 min. Once completed time treatment, 285 μL of PRP was taken and set in a CronoLog 430 aggregometer cubette with continuous stirring. Aggregation was initiated by the addition of 2×10^{-5} M adenosine diphosphate (ADP). Control group was treated with vehicle alone (ethanol <0.01%). When the specific nitric oxide synthase (NOS) inhibitor, compound L-NAME, was used it was added to the ECs in the incubation medium for 30 min prior to hormonal treatment. Immediately after the cells were washed and DMEM was replaced by PRP. Then hormonal treatment was performed and platelet aggregation was measured after the addition of 2×10^{-5} M ADP. Changes in light transmission were recorded for 5 min after ADP addition. The signal generated in platelet-poor plasma was taken as 100% transparent control. ECs were dissolved in 1 M NaOH and aliquots were taken for protein determination by Lowry Method. Results were expressed as percent of inhibition of platelet aggregation per mg protein. The maximal platelet aggregation was considered as that induced by the control samples. Two or three animals were used for each experiment.

To check the direct effect of the hormones on platelet aggregation, PRP was incubated with the steroids in the absence of ECs and platelet aggregation was measured as described above. Basal aggregation was considered the maximal aggregation exhibited by PRP alone, without vehicle or steroid treatment. Results were expressed as percent of platelet aggregation respect to basal.

2.7. Platelet adhesion to ECs

2.7.1. Preparation of platelets

PRP was obtained from whole citrated (0.38%) blood by centrifugation at 240 × g for 20 min at room temperature, and fixed in 4% formaldehyde/PBS for 10 min. PRP was suspended in PBS (pH 7.4) and centrifuged at 750 × g for 10 min. Then, fixed platelets were washed twice with PBS. Manual platelet counts were done using a hemacytometer. Finally, platelets were suspended at a concentration of 5×10^6 platelets/mL in DMEM supplemented with 1% FCS.

2.7.2. Platelet adhesion assay

Confluent monolayer of ECs seeded on 24-multiwell culture plates (NUNC) were starved for 24 h with serum-free DMEM, and then exposed to Pg, E_1 or T, LPS or hormones plus LPS in DMEM supplemented with 1% FCS. Once finished treatment, ECs were washed two times with PBS, and 300 μL of platelet suspensions were added on pretreated ECs. Platelets were allowed to adhere to ECs for 2 h at 37 °C. Supernatants of each well containing non-adhered platelets were collected and counted employing a hemacytometer. The number of adhered platelets to ECs was calculated by difference between total platelet added and non-adherent platelets. ECs and adhered platelets were also fixed with paraformaldehyde 4%, and stained using Giemsa solution. Subsequently, seven representative fields were photographed using an OLYMPUS C7070WZ optical microscope and the average numbers of adherent platelets were manually counted. Results are expressed as means ± SD of the percentage of adhered platelets respect to the number of the total platelets seeded in each well.

2.8. Measurement of NO production

NO production from ECs or from platelet was measured by Griess reaction. ECs were seeded on 24-multiwell culture plates (NUNC) at a density of 3.5×10^4 cells/well and allowed to grow to 90% of confluence in DMEM containing 10% FCS. Hormonal treatment was performed in fresh DMEM containing 1% FCS by addition of Pg, E_1 or T (or combinations) for 5 min. Respective controls (vehicle alone) were also processed. Nitrites (NO_2^-) were measured in the incubation media as a stable and non-volatile breakdown product of the NO released, employing the spectrometric Griess reaction [16]. Once finished treatment, aliquots of culture medium supernatant were mixed with Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated 10 min at room temperature. When platelet NO production was measured, PRP containing 3×10^8 platelets/mL was incubated with 1 nM T, 10 nM Pg or 10 nM E_1 for 5 min in the absence of ECs. Immediately after, platelets were centrifuged at 10,000 × g for 5 min and aliquots of supernatant were taken for NO measurement. Absorbance was measured at 548 nm in a Biotek Sinergy-HT microplate reader. The concentration of NO_2^- in the samples was determined with reference to a sodium nitrite ($NaNO_2$) standard curve performed in the same matrix. ECs or platelets were dissolved in 1 M NaOH, and protein content was measured by Lowry Method. The results were expressed as nmol of NO per mg protein.

2.9. Flow cytometry

Fresh peripheral blood samples were treated with isopropanol (vehicle) or E_1 during 24 h. Monocytes were treated with LPS for 2 h at 37 °C in 5% CO_2 atmosphere. The fluorochrome-conjugated monoclonal antibodies used were: CD11b (PE), CD11c (PE), CD14 (APC), and CD45 (PerCP). Aliquots (100 μL) of blood were labeled with the corresponding antibody for 30 min in the dark at room

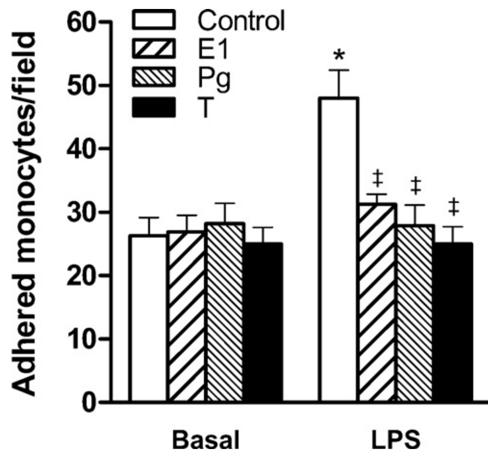

Fig. 1. Effect of E_1, Pg and T on monocyte adhesion to ECs. ECs cultures were treated with 10 nM E_1, 10 nM Pg, 1 nM T or vehicle alone (control) for 24 h in the presence or absence of 1 μg/mL LPS, added during the last 21 h of hormonal treatment. Bars represent the means ± SD of the number of monocytes adhered to ECs/field of three independent experiments ($n = 4$). $^*P < 0.001$ vs control without LPS; $^{‡}P < 0.01$ vs LPS.

temperature, and washed twice with PBS solution. Red blood cell lysis solution (BD Biosciences, San Jose, CA, USA) was added and incubated for 10 min at room temperature. Cells were processed by flow cytometry (FACS Canto II, Becton–Dickinson, USA) and data were analyzed using WinMDI 2.8 software (http://facs.scripps.edu Copyright by Joseph Trotter).

To determine whether samples were positive for each marker tested, the mean fluorescence intensity (MFI) obtained as assessed by the mean fluorescence channel was used, and compared with MFI values displayed by isotype-matched controls. Lymphocytes, monocytes and granulocytes were identified and gated according to forward scatter/sideways scatter (FSC/SSC) characteristics and levels of CD45 expression. Monocytes were discriminated with their specific marker, CD14 (gate) and the CD11b and CD11c monocytes expression was studied on this gate [20].

2.10. Statistical analysis

The results presented were obtained at least from three independent experiments where each individual experimental condition was performed by quadruplicate ($n = 4$). All data are presented as means plus its standard deviation (±SD). Different cell cultures were used for each independent experiment. Comparisons between two means were made using Student's *t*-test, and multiple comparisons with one or two ways ANOVA, followed by Fisher least significant difference test, using SSPS 10.0 for Windows. A value of $P < 0.05$ was considered statistically significant.

3. Results

In vitro treatments were performed using physiological concentrations of the hormones. On the basis of our previous dose response studies [14,16,17], we choose 10 nM for E_1 and Pg and 1 nM for T as the optimal concentrations to employ in this work.

In order to investigate the effect of E_1, Pg, and T on monocytes adhesion to ECs, cell adhesion assays were performed. LPS, bacterial endotoxin that predisposes to leukocyte recruitment and monocyte adhesion, was employed as proinflammatory stimulus. Cells were treated with E_1, Pg or T for 24 h, in the presence or absence of 1 μg/mL LPS, which was added during the last 21 h of hormonal treatment. As can be observed in Fig. 1, under basal conditions, the hormonal treatment with each steroid did not affect monocytes adhesion compared to control group. When ECs were exposed to a

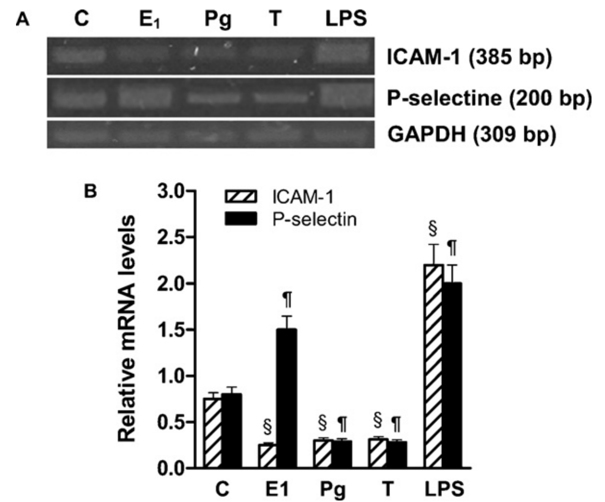

Fig. 2. Hormonal regulation of P-selectin and ICAM-1 mRNA expression. ECs were incubated with vehicle alone (control), 10 nM E_1, 10 nM Pg, 1 nM T, or 1 μg/mL LPS for 24 h. RT-PCR was performed as described in Section 2. (A) Representative gel photography of PCR amplification products. The expected band sizes for different molecule products are indicated. (B) Bars show the relative intensity of each band determined by densitometric analysis. Data are presented as P-selectin and ICAM-1 mRNA relative to GADPH mRNA and are the average ± SD of three independent experiments ($n = 4$). (C: control). $^{§}P < 0.02$ vs control I-CAM; $^{¶}P < 0.02$ vs control P-selectin.

pro-inflammatory agent (LPS), a markedly enhancement of monocytes adhesion was detected (82.5% above control, $P < 0.001$, Fig. 1). The monocyte adhesion induced by LPS was blunted in ECs exposed to E_1, Pg or T for 3 h before LPS addition.

Having in mind that monocyte and platelet adhesion to ECs is mediated by the expression of CAMs such as ICAM-1, VCAM-1 and P-selectin respectively, we evaluated the steroid regulation of mRNA expression of these CAMs. Fig. 2 shows that, LPS markedly enhanced mRNA expression of ICAM-1 compared to control group. In contrast, the mRNA levels of ICAM-1 were significantly down-regulated with respect to control group after 24 h of treatment with E_1, Pg or T (66%, 60%, and 59% of inhibition respectively). We also evaluated the effect of the steroids on VCAM-1 expression. Fig. 3 shows that mRNA levels of VCAM-1 were down-regulated with

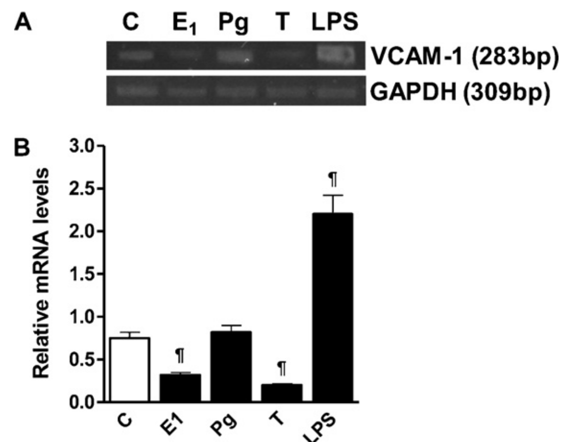

Fig. 3. Hormonal regulation of VCAM-1 mRNA expression. ECs were incubated with vehicle alone (control), 10 nM E_1, 10 nM Pg, 1 nM T, or 1 μg/mL LPS for 24 h. RT-PCR was performed as described in Section 2. (A) Representative gel photography of PCR amplification products. The expected band sizes for different molecule products are indicated. (B) Bars show the relative intensity of each band determined by densitometric analysis. Data are presented as VCAM-1 mRNA relative to GADPH mRNA and are the average ± SD of three independent experiments ($n = 4$). (C: control). $^{¶}P < 0.02$ vs control.

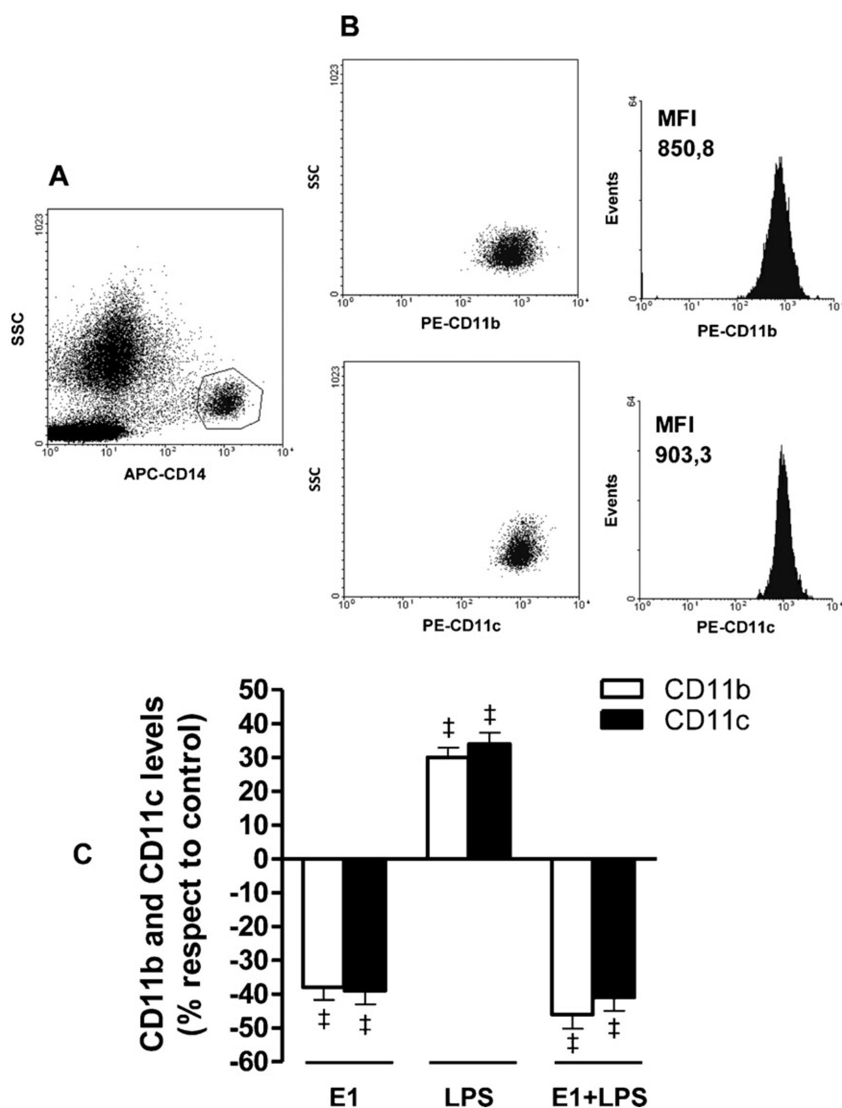

Fig. 4. Effect of E_1 on monocyte CD11b and CD11c alpha integrins expression. Monocytes were incubated with 1 μg/mL LPS for 2 h or 24 h with 10 nM E_1 in presence or absence of LPS added during the last 2 h of hormonal treatment. (A) Light scatter profiles and CD14 positivity of selected blood monocytes. (B) Dot plot and histogram of CD11b and CD11c expression of control samples. Values express the mean fluorescence intensity (MFI). (C) Quantification data expressed as % above or below control and represent the average ± SD of three independent experiments. ‡$P < 0.05$ vs control.

respect to control group after 24 h treatment with 10 nM E_1 or 1 nM T, meanwhile Pg treatment did not induce changes respect to basal levels. Similar as on ICAM-1, LPS treatment induced a two-fold increase on VCAM-1 mRNA levels.

Using FACS we examined the effect of hormonal treatment on leukocyte α-integrins expression. CD11b and CD11c bind a variety of ligands including the ICAM-1 present on endothelial cell surface. We defined monocytes as CD14+ leukocytes which were also positive for CD45, and by size determined by forward scatter. Side scatter vs CD14+ blood leukocytes showed a homogeneous population corresponding to monocytes (Fig. 4A). The analysis revealed a basal expression of CD11b and CD11c (Fig. 4B). To test the biochemical action of E_1 on the expression of both markers, blood cells were exposed 24 h to E_1. The steroid diminished CD11b and CD11c surface expression with respect to control. Monocyte treatment (2 h) with LPS significantly enhanced both adhesion molecules expression. When blood cells were treated with E_1 before LPS addition, the enhancement induced by LPS was blunted (Fig. 4C).

Then, we tested whether the hormones affect the adhesion of platelets to ECs. Cells were exposed to E_1, Pg or T for 24 h, in presence or absence of LPS. The steroids exhibited different action on basal platelet adhesion. Fig. 5A shows representative images of each individual condition. As can be observed, E_1 induced a significant increase in platelet adhesion over control group, Pg reduced the number of adhered platelets, whereas T did not alter the basal adhesion. The exposure of cells to LPS markedly increased platelet adhesion, however pre-treatment with E_1, Pg or T, completely abolished the effect induced by LPS (Fig. 5B). When the steroids effect on CAMs expression was evaluated (Fig. 2A), the results obtained on P-selectin mRNA correlates with data of basal platelet adhesion measurements. E_1 markedly enhanced the mRNA expression of P-selectin (87% above control, $P < 0.02$), while treatment with Pg or T significantly reduced its expression (Fig. 2B).

Platelet adhesion acutely stimulates aggregation process. We studied the effect of hormonal treatment on endothelium dependent platelet aggregation after a short time of exposure to the steroids. ECs were incubated in PRP and then treated with T, Pg or E_1 for 10 min. Fig. 6 shows that the three steroids significantly inhibited platelet aggregation (white bars). To check the involvement of NO, the NOS inhibitor L-NAME was employed. In the presence of L-NAME (black bars), the inhibition of platelet aggregation induced by T and Pg was partially suppressed, while the inhibitory effect

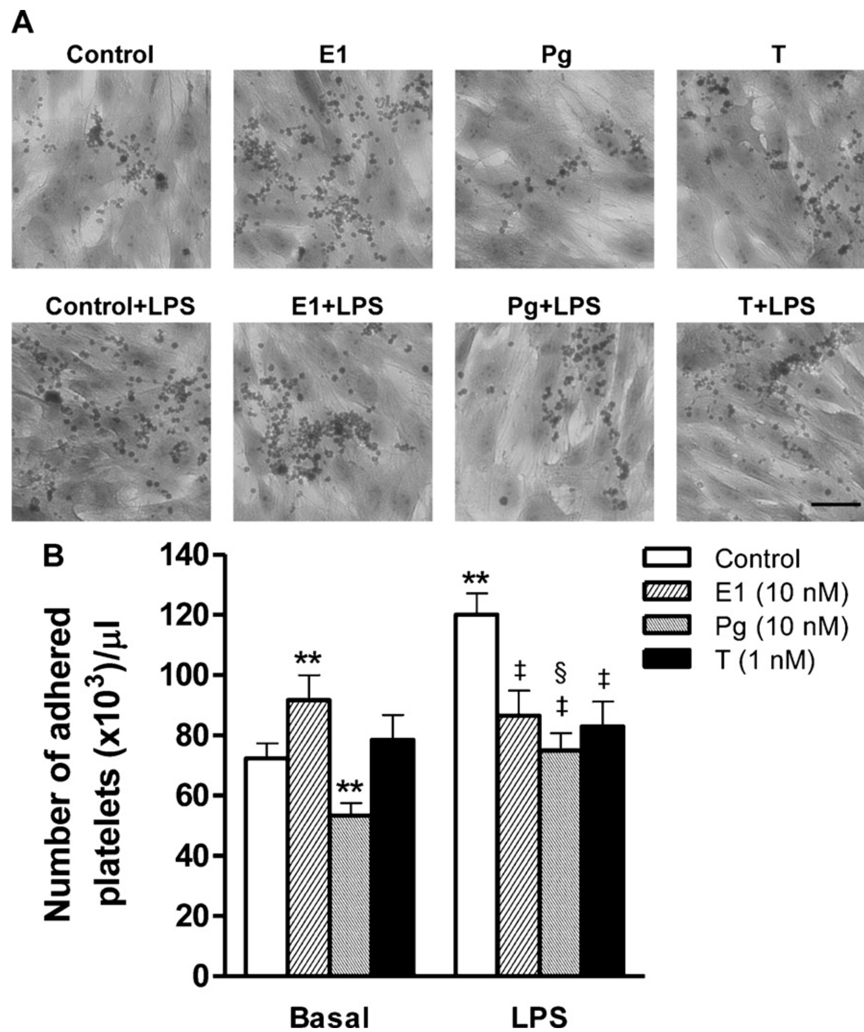

Fig. 5. Effect of E_1, T and Pg on platelet adhesion to ECs. ECs cultures were treated with 10 nM E_1, 10 nM Pg, 1 nM T, or vehicle alone (control) for 24 h in the presence or absence of 1 µg/mL LPS, added during the last 21 h of hormonal treatment. Platelet adhesion was measured as described in Section 2. (A) Images (200×) show representative fields of each experimental condition (scale bar represents 70 µm). (B) Bars represent the means ± SD of adhered platelets counted/µL of three independent experiments ($n = 4$). $^{**}P < 0.02$ vs control without LPS; $^{§}P < 0.02$ vs Pg; $^{‡}P < 0.02$ vs LPS.

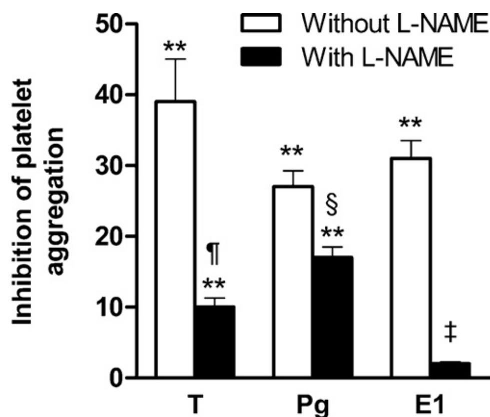

Fig. 6. Inhibition of platelet aggregation induced by T, Pg, and E_1. Confluent ECs were preincubated for 30 min with (black bars) or without (white bars) 10 µM L-NAME. DMEM was replaced by PRP and cells were exposed to 1 nM T, 10 nM Pg, or 10 nM E_1 for 10 min. Platelet aggregation was measured as described in Section 2. Results are expressed as percent of inhibition of platelet aggregation respect to each control group (ECs plus vehicle alone) and are the average ± SD of three independent experiments ($n = 4$). $^{**}P < 0.02$ vs control; $^{§}P < 0.001$ vs Pg; $^{‡}P < 0.001$ vs E_1; $^{¶}P < 0.001$ vs T.

Table 1
Effect of steroid treatment on platelet nitric oxide production and on platelet aggregation.

Treatment	NO production (nmol/mg protein)[a]	Platelet aggregation (% respect to basal)[b]
Control	0.75 ± 0.06	97 ± 3.5
1 nM T	0.77 ± 0.07	98 ± 5.8
10 nM Pg	0.78 ± 0.06	99 ± 7.3
10 nM E1	0.80 ± 0.07	97 ± 7.8

Aliquots of PRP containing 3×10^8 platelets/mL were incubated with T, Pg or E_1 at the indicated concentrations for 5 min. Control group received vehicle alone.

[a] NO release to the incubation medium was measured as described in Section 2.

[b] Aliquots of PRP were placed in the aggregometer and ADP-induced platelet aggregation was measured. Data are presented as % of platelet aggregation respect to basal (PRP without treatment). Results are the average ± SD of three independent experiments ($n = 4$).

exerted by E_1 was completely abolished, suggesting that the anti-aggregatory effect induced by the steroids is partially or completely dependent on the NO released from ECs. We also considered the possibility of a direct action of the steroids at platelet level. To that end, platelet aggregation in the absence of ECs was measured. As can be observed in Table 1, the addition of T, E_1 or Pg to PRP without the presence of ECs induced maximal aggregation similar to

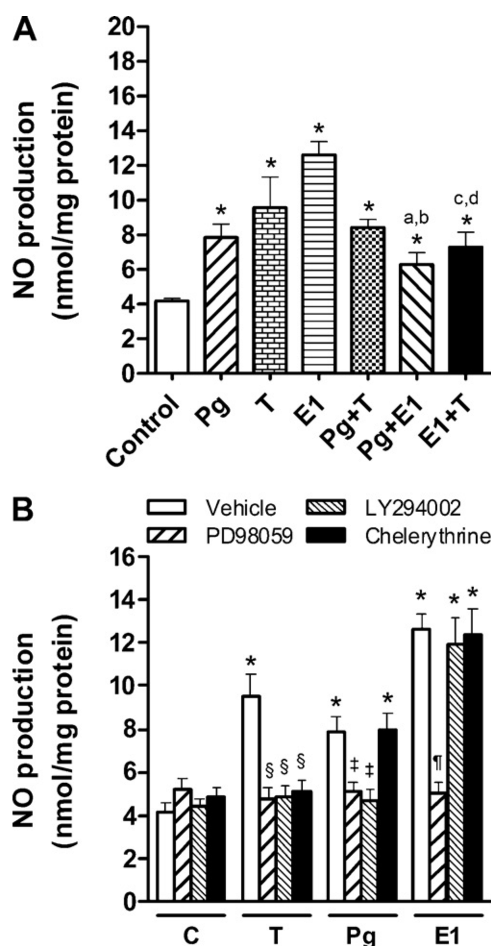

Fig. 7. Effect of hormonal treatment on endothelial NO production. (A) Starved ECs were treated with 10 nM Pg, 1 nM T or 10 nM E_1 for 5 min an NO was measured by Griess reaction as described in Section 2. $^{*}P<0.001$ vs control; $^{a}P<0.05$ vs Pg; $^{b}P<0.01$ vs E_1; $^{c}P<0.01$ vs E_1; $^{d}P<0.05$ vs T. (B) ECs were pre-incubated in the absence (vehicle) or presence of 5 μM PD98059, 1 μM LY294002 or 1 μM chelerythrine for 30 min, and then exposed to steroids for 5 min. NO production was measured by Griess reaction. Results represent the average ± SD of three independent experiments ($n=4$). $^{*}P<0.001$ vs control; $^{\S}P<0.02$ vs T; $^{\ddagger}P<0.02$ vs Pg; $^{¶}P<0.02$ vs E_1.

basal group, excluding a direct effect of the steroids on platelet aggregation.

Taking in account our previous data about the fast action of each steroid on rat aortic strips NO synthesis, we tested the effect of combined treatment with the steroids on the regulation of NO production by isolated ECs. Short time intervals of hormonal treatment (5 min) were employed. Fig. 7A shows that the three hormones significantly enhanced NO synthesis when added separately. Simultaneous addition of Pg and T also induced a marked stimulation in the vasoactive synthesis (101% above control, $P<0.001$) of similar magnitude to that obtained with Pg or T alone. However, although combined treatment of Pg plus E_1 enhanced NO production (50% above control, $P<0.001$), the increase was lower than the effect observed for Pg or E_1 treatment alone. Similar results were obtained when the combined treatment were performed with of E_1 plus T. In addition, the direct effect of hormonal treatment on platelet NO production was also investigated. To that end, aliquots of PRP were exposed to T, Pg or E_1 for 5 min, and NO release to the incubation medium was measured. Table 1 shows that no significant differences in platelet NO production between control and treated group were obtained.

It is known that PKC, MAPK, and phosphatidylinositol 3-kinase (PI3K) signaling pathways are involved in NOS stimulation.

Table 2
Effect of NOS inhibitors on monocyte adhesion.

Treatment	Monocyte adhesion (adhered monocytes/field)[a]		
	Without inhibitors	+L-NAME	+L-NIL
Control	26.3 ± 2.9	25.3 ± 3.1	27.3 ± 3.5
1 nM E1	26.3 ± 2.6	22.8 ± 2.1	23.9 ± 3.3
10 nM Pg	28.2 ± 3.2	25.8 ± 3.4	27.8 ± 3.3
10 nM T	25.0 ± 2.6	25.9 ± 2.9	22.9 ± 2.9
LPS	48.3 ± 4.4*	35.4 ± 2.8‡	37.8 ± 4.2‡‡
1 nM E1 + LPS	31.3 ± 1.6	28.5 ± 2.6	29.5 ± 5.6
10 nM Pg + LPS	27.9 ± 3.2	22.4 ± 1.4	27.4 ± 2.1
10 nM T + LPS	25.3 ± 2.7	27.6 ± 3.0	25.6 ± 2.9

* $P<0.001$ vs control

[a] ECs cultures were preincubated with 10 μM L-NAME or 50 μM L-NIL for 1 h, and immediately after, treated with 10 nM E_1, 10 nM Pg, 1 nM T or vehicle alone (control) for 24 h in the presence or absence of 1 μg/mL LPS added during the last 21 h of hormonal treatment. Results represent the means ± SD of the number of monocytes adhered to ECs/field of three independent experiments ($n=4$). $^{\ddagger}P<0.05$ vs control + L-NAME; $^{\ddagger\ddagger}P<0.05$ vs control + L-NIL.

Therefore, we examined whether the regulation of NO production by E_1, T, and Pg, involves the participation of these intracellular signaling systems. To that end, ECs were incubated in presence or absence of LY294002 (PI3 K inhibitor), PD98059 (MAPK inhibitor) or chelerythrine (PKC inhibitor) before treatment with 10 nM E_1, 10 nM Pg or 1 nM T. Pretreatment of cells with MAPK inhibitor completely abolished the stimulatory effect on NO synthesis exerted by E_1, T or Pg. Inhibition of PI3K with LY294002 did not alter the stimulation induced by E_1 but completely suppressed the stimulation of NO production elucidated by treatment with Pg or T. The presence of chelerythrine only affected the stimulatory action induced by T. The presence of the PKC inhibitor abolished the enhancement in NO elicited by the androgen (Fig. 7B).

Finally, we evaluated whether NO could mediate the inhibitory action of the steroids on monocyte adhesion induced by LPS. To that end, L-NAME and L-NIL were selected as NOS and iNOS inhibitors respectively. ECs were preincubated with 10 μM L-NAME or 50 μM L-NIL for 1 h before the exposure to E_1, Pg or T for 24 h. LPS was added during the last 21 h of hormonal treatment, and monocyte adhesion was measured as described in Section 2. Table 2 shows that, both inhibitors partially reduced monocyte adhesion induced by LPS (82% vs 40–38% above control; LPS vs LPS + inhibitors respectively). The steroids prevented the increase of monocyte adhesion induced by LPS, and the presence of the inhibitors did not affect the inhibitory action elicited by the hormones. The steroids completely suppressed the adhesion induced by LPS even though the presence of L-NAME or L-NIL. These results suggest that, although the inflammatory conditions could affect NOS balance, when the steroids are present before LPS treatment the hormones are capable to blunt monocyte adhesion induced by LPS, in a NO independent manner.

4. Discussion

The results reported in this work provide evidence that the sex steroids E_1, Pg, and T modulate monocytes and platelets adhesion to ECs, events involved in the initiation and progression of vascular lesions. The steroids actions were evidenced under basal or inflammatory conditions. As cell adhesion is mediated through CAMs, we also found that the hormones regulate CAMs expression in monocyte and ECs. Furthermore, we showed that the steroids inhibited endothelial dependent platelet aggregation through their direct action on the vascular endothelium prompting NO production. The molecular mechanisms of action exerted by the steroids included the selective and differential participation of the intracellular signaling pathways PKC, MAPK, and PI3K, on NO release stimulation.

In healthy conditions, the endothelium remains in a non-activated state. The initial response to vascular injury includes activation, adhesion, and aggregation of platelets to endothelium. Platelets release cytokines promote monocytes recruitment and their activation to macrophages [21]. ICAM-1 is one of the CAMs implicated in the early steps of transendothelial cell migration process. Interaction of ICAM-1 with activated integrins ensures stable monocyte adhesion to the endothelium, and thereby enables them to migrate to the underlying tissue [23]. After the initial leukocyte attachment and rolling, VCAM-1 triggers monocyte transendothelial migration to the intima [24]. Expression of ICAM-1 gene in ECs can be promoted by a variety of mediators (thrombin, TNF-α, IL-1β, and LPS) that induce oxidative stress and stimulate CAMs genes encoding transcription [22]. In this work we showed that, under basal conditions the hormonal treatment did not affect monocyte adhesion to ECs. However, in the presence of LPS the number of monocytes adhered to ECs was significantly reduced when E_1, Pg, or T were added prior to LPS, suggesting that the three steroids are able to prevent monocyte adhesion induced by inflammatory stimulus. Although the inflammatory conditions could affect NOS balance, the inhibitory action of the steroids was independent of NO. As was expected LPS treatment induced a pronounce increase in ICAM-1 and VCAM-1 expression. In contrast, the steroids reduced the expression ICAM-1 and VCAM-1 mRNA. It has been reported that in human vein endothelial cells (HUVECs), Pg inhibited VCAM-1 and ICAM-1 expression [11]. Indeed aortic macrophage infiltration was diminished in rat in vivo supplemented with androgens [25].

We also demonstrated that in blood monocytes, E_1 treatment reduced surface expression of CD11b and CD11c integrins, and also prevented LPS induction. Sex steroids regulate vascular inflammatory environment modulating CAMs expression on ECs and monocytes. It has been recently shown that LPS-induced CD11b-expression on granulocytes is regulated by estradiol, Pg or both [26].

To study the interactions between platelets and ECs we evaluated both, the adhesion and the aggregation process. Under basal conditions each steroid exhibited an individual adhesion profile. Basal platelet adhesion was increased after E_1 treatment whereas Pg diminished it, and T did not induce significant changes. These observations were consistent with the effect of the steroids on mRNA P-selectin expression, which was enhanced by E_1 treatment and reduced after T or Pg exposure. Once more, we found that in an inflammatory environment the increase in platelet adhesion induced by the bacterial endotoxin LPS was prevented by the presence of the steroids.

It is known that platelet adhesion triggers platelet aggregation. The aggregation process is modulated by NO and the eicosanoids prostacyclin and thromboxane. Moreover, NO is the main regulator of vascular homeostasis that induces vasodilation and inhibits CAMs expression, monocytes and platelets adhesion and aggregation [27–29]. We provided evidence E_1, Pg and T exhibited a markedly antiaggregatory action and that NO was involved in the inhibition of platelet aggregation induced by the hormones, since the presence of NOS inhibitor L-NAME decreased the antiaggregatory effect. A direct effect of the hormonal treatment on platelet activity was ruled out since in the absence of ECs, platelet aggregation was maximal. Moreover, platelet NO production was not affected by short treatment with the steroids.

In NO measurements we showed that, although NO production was markedly enhanced when ECs were treated separately with each hormone, simultaneous addition of two steroids modulates NO release with different pattern respect to each individual effect. If the dual treatment includes E_1 (E_1 + Pg or E_1 + T), the combined treatment exhibited a lower effect than Pg, T or E_1 alone. The mechanisms of action of the steroids in rat aortic tissue previously reported, include signal transduction pathways activation with direct stimulation of phospholipase C (PLC), PKC, and MAPK activities [14,16,19]. In view of this, we evaluated the participation of MAPK, PKC, and PI3K in the hormonal regulation of NO production. We found that each steroid modulates NO production through the selective activation of specific signaling cascades. The results obtained using the kinase inhibitors suggest that the enhancement in NO release induced by E_1 involves MAPK cascade, meanwhile the raise induce by Pg is mediated by MAPK and PI3K and stimulatory action of T involves the participation of the three signaling pathways evaluated. Therefore, a possible explanation for the differences observed in combined steroid treatments could be attributed to a possible cross-talk between the different signaling pathways activated by each hormone. The mechanism of endothelial NOS activation by E_2 has been extensively studied. It has been reported that PI3K and MAPK signal transduction pathways mediate NOS regulation by E_2 [30]. In agreement with our results, inhibition of MEK1/2 kinases suppresses the stimulation of NO synthesis induced by Pg in HUVECs [11]. Indeed in human ECs, T increase endothelial synthesis of NO through a rapid activation of the extracellular-related kinase (ERK) 1/2 and PI3K/Akt cascades [31]. In HUVECs, T induces MAPK, ERK1/2, and MEK1/2 phosphorylation within a short time [32].

Bearing in mind that during menopause the circulating levels of E_1, Pg, and T change, the major risk of cardiovascular disease observed in postmenopausal women might be attributed in part, to alterations on the vascular steroid action. In view of the central role of NO on vascular homeostasis, we could speculate that the impairment in the ability to produce NO when the steroids are present simultaneously could lead to prompt vascular dysfunction. Since the results present in this work belong from in vitro assays using isolated cells at the present we do not know their physiological relevance.

5. Conclusions

In summary, we provided evidence that, the three steroids are able to prevent endothelial injury induced by LPS. Under basal conditions, each hormone exhibits its own mechanism with distinct features, but in an inflammatory environment they elicit similar effects. Although the pathophysiological significance of our results is still unknown, these data might be useful for a better comprehension of biochemical action of sex steroids on vascular behavior, and in future, could contribute to provide molecular basis to design alternative protocols of replacement therapies in order to improve vascular health.

Conflict of interest

None declared.

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