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Cellular and molecular actions displayed by estrone on vascular endothelium

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ABSTRACT

In this work we provide evidence that estrone "per se" modulates cellular endothelial growth and survival, events that play key roles in the development of vascular disease. Moreover, under oxidative stress conditions the hormone prevented apoptosis triggered by hydrogen peroxide. Although estrone did not affect E-selectin and VCAM-1 mRNAs synthesis, the hormone prevented the expression of these adhesion molecules induced by the proinflammatory agent LPS. The steroid partially attenuated leukocyte adhesion not only under basal conditions but also in the presence of LPS. Using ICI182780 compound as estrogen receptor antagonist, and PD98059 as MAPK inhibitor we obtained evidence that the mitogenic action of estrone involved the participation of ER and MAPK transduction pathway activation. The presence of estradiol impaired the effect of estrone on cell proliferation and vasoactive production. These results suggest that estrone exhibits a remarkable biological action on endothelial cells, modulating vasoactive production, proliferation, apoptosis, and cell adhesion events.

1. Introduction

Estrogens play an important role in reproduction and sexual development, as well as in the regulation of cellular events on different target tissues. Estrone (E_1) is a physiological estrogen previously categorized as weak estrogen. However, in the last few years, several biochemical actions of E_1, either in classical and non classical estrogen target tissue (pituitary, breast, vascular, colon) have been described (Watson et al., 2008; Motylewska and Melén-Mucha, 2009). Although estradiol (E_2) is the endogenous estrogen most often associated with female reproduction and with postmenopausal alterations and diseases, other physiological estrogens such as estrone or estriol also seem to be relevant during specific life phases, eliciting significant effects on tissue development, function and diseases. E_1 displays several actions both in reproductive and postmenopausal women. After menopause, E_1 represents the major source of circulating estrogen. Although its ovarian contribution declines, the steroid production is sustained via androstenedione aromatization in adipose tissue (Bulun et al., 1999; Grow, 2002). Moreover, in hormone replacement therapy, estrone represents the major estrogen provided by conjugated equine estrogens (Notelovitz, 2006). The difference in cardiovascular disease incidence between men and fertile women is mainly attributed to the distinctive level of plasmatic sex hormones and the presence of specific sex risk factors. In postmenopausal women the prevalence of atherosclerosis increases. This fact was associated with the loss of atheroprotective effects exerted by estrogens, particularly by E_2 (Mendelsohn and Karas, 1999). Most studies reported in the literature focused on estradiol impact both in fertile and/or postmenopausal women, however little is known about the cellular and molecular action of estrone.

Estradiol produces multiple actions on vascular tissue such as regulation of vasoactive compounds production, or modulation of target proteins synthesis involved in cell proliferation, differentiation, apoptosis, and migration (Mendelsohn and Karas, 1999; Meyer et al., 2009). These events require estradiol binding to estrogen receptors (ERs) and involve the participation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-OH kinase (PI3K), small GTPase RhoA and other intracellular signal transduction pathways (Simoncini, 2009; Geraldes et al., 2002, 2003; Pedram et al., 2006).

In the last decade, the actions of steroid hormones have been classified into two types: those called "genomic" effects, associated with the regulation of gene transcription, and those identified as "non-genomic" effects, characterized by a rapid onset activation of the signal transduction pathways, and of short duration (Falkenstein et al., 2000; Pedram et al., 2002).

We had previously reported that, at vascular level, E_1 produces genomic and non genomic effects. Rapid actions involve NOS and COX activation and inhibition of platelet aggregation in a NO dependent manner (Massheimer et al., 2003; Sellés et al., 2005). The action mechanism displayed by E_1 differs from that reported

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for estradiol (Miller and Duckles, 2008). In rat aortic strips, the non-genomic action of E_1 includes PLC/DAG transduction pathway activation and a cross talk between NOS and COX systems (Massheimer et al., 2006). PI3K transduction system does not mediate the steroid rapid action on nitric oxide synthesis. Indeed, in isolated vascular smooth muscle cells (VSMC), we demonstrated that E_1 promotes cell proliferation and migration through PKC transduction system activation (Rauschemberger et al., 2008).

Vascular endothelium has a pivotal role in haemostasis regulation, vascular tone, endothelial permeability and vascular cell growth. Changes in any such endothelial properties induced by abnormal stimuli can result in cellular or molecular alterations that may affect vessel health. These alterations may induce changes in cell proliferation, apoptosis, expression of adhesion molecules, synthesis of vasoactive compounds mainly nitric oxide and prostacyclin. The integrity of the vascular layer depends on a tight regulation of those cellular events either at endothelial or vascular smooth muscle cellular level. Endothelial cells (EC) proliferation and survival are particularly important in angiogenesis, vascular repair and in prevention of atherosclerosis disease. In healthy conditions, EC have a low proliferation rate, but when the endothelial layer is damaged, vessel repair is achieved by migration and proliferation of adjacent EC (Cartwright et al., 2000). In atherosclerosis, EC turnover and apoptosis are altered (Choy et al., 2001). After endothelial damage, plaque development starts with leukocyte recruitment to the arterial vessel wall and subsequent tethering, rolling, firm adhesion, and transmigration of monocytes to the subendothelial layer. When the lesion progresses, it results in loss of endothelial cells.

Having in mind the pivotal role that EC exert in vascular homeostasis, the aim of the present work was to evaluate whether endothelial cellular growth, apoptosis, and cellular adhesion could be regulated by the natural ovarian steroid, estrone. The molecular action mechanism of the hormone was also studied.

2. Materials and methods

2.1. Materials

Estrone, PD 98059, Equilin, Chelerytrine, DULBECCO'S modified EAGLE'S medium, lipopolysaccharides (LPS from *Escherichia coli* 0127) were purchased from Sigma Chemical Co. (St. Louis, USA); ICI 182780 from Tocris Bioscience (Park Ellisville, USA). Griess reaction solutions were obtained from Britania Laboratories (Buenos Aires, Argentina) and Trypsin/EDTA (10×), L-glutamine (100×), amphotericin B (0.25 mg/mL), penicillin/streptomycin (100×) and Foetal Calf Serum (FCS) from PAA Laboratories (Pasching, Austria). All reagents for PCR and RT-PCR were obtained from Invitrogen (California, USA) and PROMEGA (USA).

2.2. Rat aortic endothelial cell cultures

Endothelial cells were obtained from aortic ring explants isolated from young Wistar female rats (3–5 weeks old) as described previously (Cutini et al., 2009). Briefly, the full length thoracic aorta was aseptically removed, and then cut into ring segments (1–1.5 mm). Ring explants were seeded in a 60-mm matrix-coated petri-dishes (NUNC) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin-B, and 2 mM L-glutamine, and 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. EC identity (Bachetti and Morbidelli, 2000) was determined, (a) by phase-contrast microscope observation of the characteristic morphology of cobblestone shape growth in confluent monolayers, (b) by the positive immunocytochemistry reactivity to Factor VIII, and to anti-Vimentin, clone V9 using DakoCytomation EnVision system, and (c) by NO synthesis ability. Cells from passages 2–7 were used for all experiments. All animal work was performed at the Unit of Animal Care belonging to the Biology, Biochemistry and Pharmacy's Department of the University. The Animal Care Use Committee approved the protocol used.

2.3. Measurement of NO production by Griess method

Endothelial cells were seeded on NUNC 24 multi-well plates at a density of 3.5×10^4 cells/well, and allowed to grow to 90% confluence. Hormonal treatment was performed by the addition of the steroids to fresh DMEM containing 1% FCS. Immediately after treatment, 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. Briefly, aliquots of culture medium supernatant were mixed with Griess reagent and incubated 10 min at room temperature. Absorbance was measured at 548 nm in a Hitachi U-1000 spectrophotometer. Nitric oxide concentration was determined with reference to a sodium nitrite standard curve performed in the same matrix. Cells were dissolved in 1 M NaOH, and taken aliquots for protein determination by Lowry method. The results were expressed as nmol of NO_2^- per mg protein (Sandoval et al., 2010).

2.4. Determination of [^{3}H]-thymidine incorporation

Subconfluent EC that were cultured in 12-well culture plates were made quiescent by serum deprivation for 1 day. The media was changed to DMEM plus 1% serum and the monolayers were exposed to E_1 (10 nM), E_2 (10 nM) or $E_1 + E_2$ for 24 h. During the last hour of treatment the cells were pulsed with 1 μCi/ml of [^{3}H]-thymidine. When the compounds Equilin (Eq), ICI182780, PD98059 or Chelerythrine (Chel) were used they were added 30 min prior hormonal treatment. Cells were rinsed twice with PBS to remove the unincorporated [^{3}H]-thymidine. Ice-cold trichloroacetic acid (10%) was added and the acid-insoluble material was solubilized with NaOH 1 M. Radioactivity was measured by liquid scintillation, and [^{3}H]-thymidine incorporation per well was normalized to protein content (Kyaw et al., 2002).

2.5. DNA fragmentation assay

EC cultured in DMEM containing 1% FCS were exposed to E_1 or vehicle alone. H_2O_2 (200 μM) was employed as apoptosis inducer, and was added during the last 6 h of hormonal treatment. Cells were washed twice with PBS and lysated with 10 mM EDTA, 400 mM NaCl, 1 mg/ml proteinase K, 35 mM SDS and 10 mM Tris–HCl pH 8.2 at 37 °C overnight. DNA was extracted using phenol–chloroform–isoamyl-alcohol (25:24:1). DNA samples were separated by electrophoresis on 1% (w/v) agarose gel containing 1 mg/ml of ethidium bromide and photographed on an ultraviolet transilluminator (Ohtsuka et al., 2006). Integrity density was measured employing the image processing and analysis software Image J (1.43 c version, NIH, Rasband).

2.6. Cells adhesion assay

Monocyte (PBM) isolation was performed by densitiy gradient (Ficoll-Paque Plus) and subsequent cultured in DMEM plus 10% FCS as previously described (Sandoval et al., 2010).

To carry out PBM adhesion assays EC were starved for 24 h with serum free medium, and then exposed to 10 nM E_1 in presence or absence of LPS (1 μg/ml), in DMEM (1% FCS). An exact number of PBM was seeded on pretreated EC and incubated during 2 h at 37 °C humidified 5% CO_2 atmosphere (Pawlowski et al., 1985). Bound monocytes were counted (3 fields/ well). EC and adhered PBM were dyed using Giemsa stain. Images (200×) were obtained using an OLYMPUS C7070WZ optical microscope system. Results are expressed as means ± SD of number of cells counted.

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

Expression of the EC adhesion molecules, E-selectin, and VCAM-1 was determined using the Superscript III CellsDirect cDNA synthesis system (Invitrogen, California, USA). To that end, endothelial cells (grown in 12-well culture plates) were treated for 24 h with 10 nM E_1 in the presence or absence of LPS employed as an inductor of adhesion molecule expression. Cells were washed twice with ice-cold PBS and total cellular RNA extraction and RT-PCR were performed according to the manufacturer's instructions. Complementary DNA was amplified using a programmed Thermocycler (BIOMETRA UNO II, Goettingën, Germany) (Sandoval et al., 2010). PCR cycles were as follows: E-selectine (95 °C, 3 min, 94 °C, 30 s, 53 °C, 45 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 for E-selectine were: forward: 5′CAA CGT GCA CGT TTG ACT GT 3′, reverse: 5′AGG TCA AGG CTT GAA CAC TG 3′; for VCAM-1 were: forward: 5′ TAA GTT ACA CAG CAG TCA AAT GGA 3′ (Peng et al., 2006; Callera et al., 2004). The expression of housekeeping gene GAPDH (glycerldehyde-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 without RT product) were also processed. PCR amplification products were detected by electrophoresis in agarose gels stained with ethidium bromide. Results were obtained from at least 3 independent experiments. The density of each band on RT-PCR gel was quantified using ImageJ software (1.43 c version, NIH, Rasband). The mRNA in each sample was normalised against the quantity of GAPDH mRNA.

2.8. Statistical analysis

Each experimental condition has been performed in three independent experiments performed by quadruplicate. All data are presented as average ± SD.

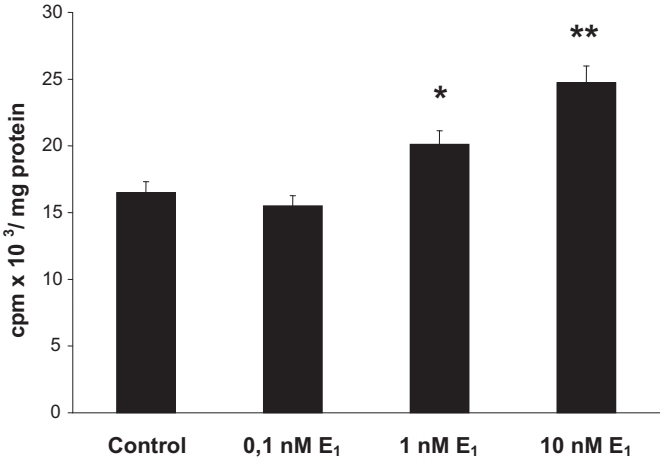

Fig. 1. Effect of estrone on endothelial cells proliferation. Dose–response profile. Starved EC cultures were incubated with the indicated concentrations of E_1 for 24 h. [^{3}H]-thymidine incorporation was measured as described in Section 2. Results represent the average ± SD of three independent experiments ($n = 4$). $^*p < 0.01$; $^{**}p < 0.001$ respect to control value.

Comparisons between two means were made using Student's *t*-test, and multiple comparisons with ANOVA using SSPS Statistical software version 10.0 for Windows. Differences of $p < 0.05$ were considered significant.

3. Results

Using ^{3}H-thymidine incorporation assay, the effect of E_1 on EC growth was evaluated in dose and time response studies. As can be observed in Fig. 1, 1 and 10 nM E_1 significantly increased DNA synthesis. Lower concentration did not affect cellular growth. The mitogenic action of the hormone was detected after 24 h of treatment (16158 ± 1216 vs. 24075 ± 1708 cpm/mg protein, C vs. 10 nM E_1 respectively, $p < 0.001$). Lesser stimulation of EC proliferation was observed after 36–48 h of steroid treatments (25; 15% increase in DNA synthesis, $p < 0.02$). After 72 h of hormonal treatment, no significant differences between control and E_1 groups were detected (16158 ± 1216 vs. 17127 ± 1524 cpm/mg protein, C vs. 10 nM E_1 respectively). These effects were confirmed by cellular counting after Giemsa EC staining.

To assess the effect of E_1 on programmed cell death, we chose DNA fragmentation as a feature of apoptosis. Fig. 2 shows that the hormonal treatment did not affect EC apoptosis since no laddering pattern was observed in DNA extracted from EC exposed to 10 nM E_1. In contrast, the apoptotic inductor H_2O_2 provoked a complete DNA laddering compared to control group. When E_1 was added prior to 200 μM H_2O_2 treatment, the apoptotic effect of H_2O_2 was clearly attenuated. These results suggest that apoptotic stage induced by oxidative stress would be partially prevented by E_1.

It is known that endothelial surface is target for monocytes adhesion and transmigration. Under basal conditions, few monocytes adhered to the endothelium, but adhesion is markedly increased after injury. The effect of E_1 on PBM adhesion to EC was measured using cell adhesion assay. EC were treated with 10 nM E_1 for 24 h, and immediately after isolated PBM were added. The microphotography presented in Fig. 3 shows that E_1 inhibited adhesion since lesser amount of monocytes was adhered to EC compared to control group. EC treatment (20 h) with the proinflammatory agent LPS (1 μg/ml) significantly enhanced the number of monocytes adhered to EC. Monocyte adhesion induced by LPS was partially attenuated when EC were treated with 10 nM E_1 prior bacterial LPS addition.

It is known that monocyte adhesion to EC is mediated by the presence of cellular adhesion molecules (CAMs) on the luminal side of endothelial cells. We detected that, in our experimental system, LPS enhanced mRNA expression of E-selectin and VCAM-1 adhesion molecules compared to control group (1st vs. 2nd lane, Fig. 4). To test the biochemical action of E_1 on the transcriptional regulation of these adhesion molecules, EC cultures were exposed 24 h to 10 nM E_1. The steroid did not affect E-selectin mRNA levels and reduced VCAM-1 transcription respect to control group. However, the enhancement in mRNA of both CAMs induced by LPS was down regulated in presence of E_1. This effect was observed either when 10 nM E_1 was added prior or after LPS treatment.

The mechanism of action displayed by the hormone was also studied. Since E_1 may be converted in estradiol through the catalytic action of 17-beta-hydroxysteroid dehydrogenase 1 (17-β-HSD1), we used the Equilin compound, as 17-β-HSD1 inhibitor. EC were treated with E_1 for 24 h in absence or presence of 1 or 10 μM Equilin. As can be observed in Fig. 5, the presence of the inhibitor did not affect the mitogenic action induced by E_1. No sta-

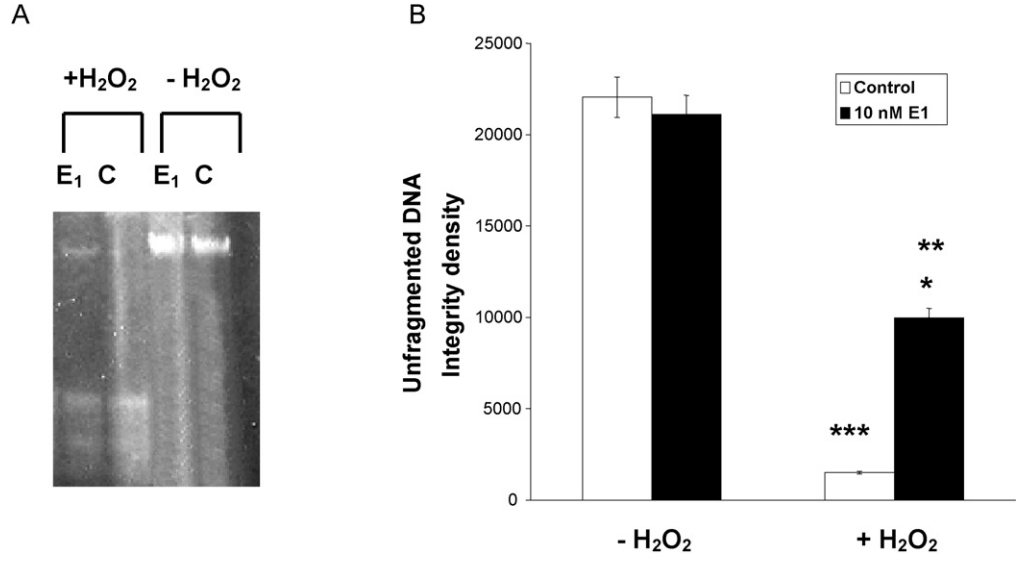

Fig. 2. Regulation of endothelial cells apoptosis by estrone. EC cultures were treated with 10 nM E_1 or vehicle alone (control) for 24 h. When 200 μM H_2O_2 was employed it was added during the last 6 h of treatment. DNA fragmentation was detected by electrophoresis as described under Section 2. A representative image (A) and results of integrated density of unfragmented DNA (B) from three independent experiments are shown. $^*p < 0.001$ vs. H_2O_2, $^{**}p < 0.001$ vs. E_1, $^{***}p < 0.001$ vs. control.

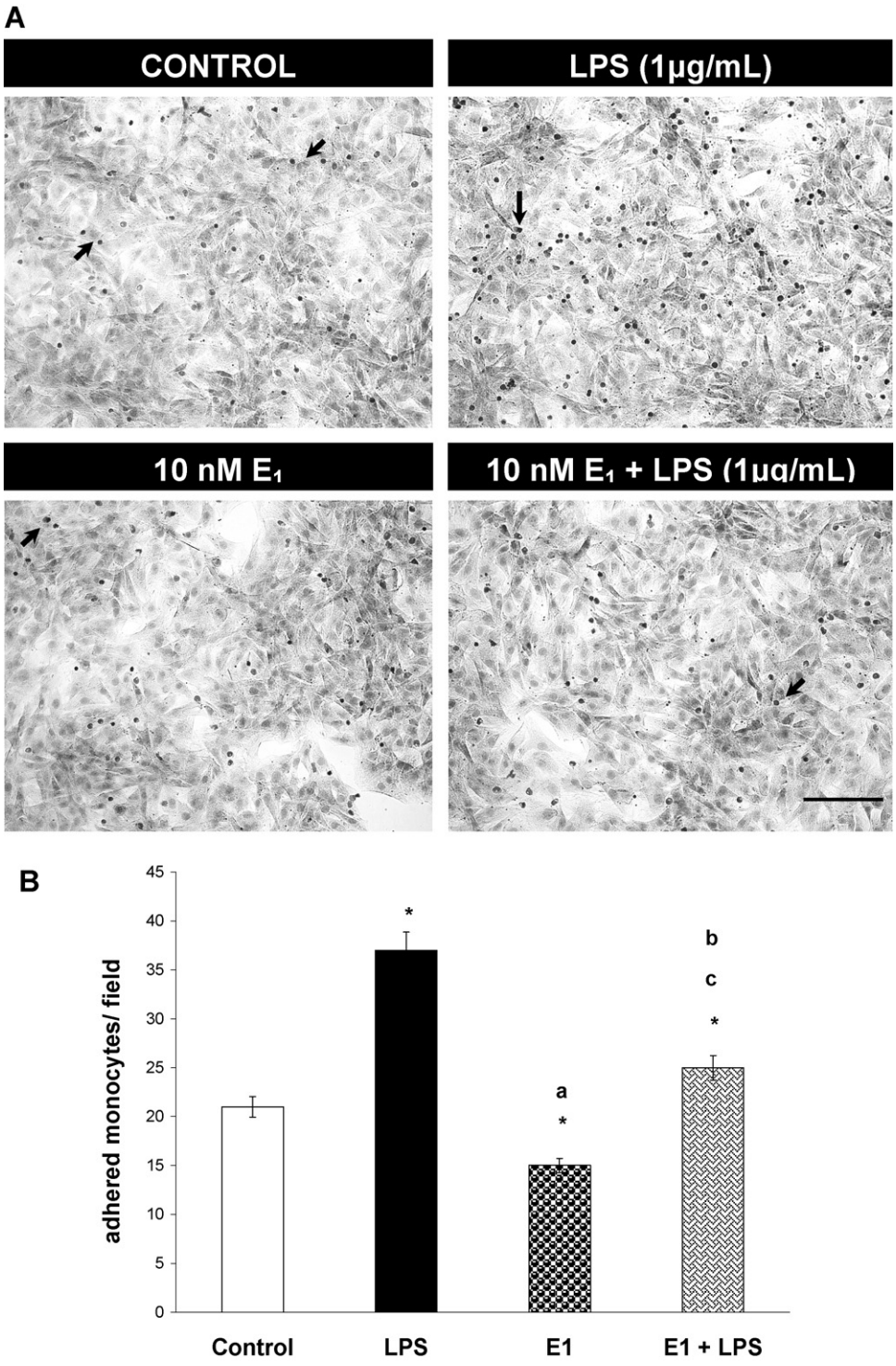

Fig. 3. Effect of E_1 on monocyte adhesion to endothelial cells. EC cultures were treated with 1 μg/ml LPS for 20 h; or 10 nM E_1 for 24 h in the presence or absence of 1 μg/ml LPS added during the last 5 h of hormonal treatment; or vehicle alone (control). PBM were seeded on EC monolayer for 2 h, and immediately after the supernatant was removed and the adhered PBM/ field were counted. (A) Images show representative fields of each experimental condition (200×). The scale bar represents 70 μm. Black arrows show monocytes adhered to EC. (B) Bars represent the means ± SD of the number of monocytes adhered to EC/field. Results are the average ± SD of three independent experiments ($n = 4$). *$p < 0.001$ vs. control; (a) $p < 0.001$ vs. LPS; (b) $p < 0.001$ vs. LPS; (c) $p < 0.001$ vs. E_1.

tistically differences ($p > 0.10$) were observed between E_1 alone and E_1 + Equilin (1 or 10 μM).

To assess whether ER would mediate E_1 actions, we evaluated the steroid effect on DNA synthesis in presence of the ER antagonist, ICI182780. Fig. 6 shows that the pure antiestrogen effectively blocked ^{3}H-thymidine incorporation induced by E_1 (93 vs. 8% stimulation above each control, E_1 vs. E_1 + 10 μM ICI182780, $p < 0.001$). Having in mind that MAPK cascade is involved in the cellular growth regulation, we evaluated the participation of this transduction system using PD98059 compound (MEK inhibitor). When EC cultures were preincubated with 5 μM PD98059 prior to 10 nM E_1 treatment, the proliferative effect exerted by the hormone was suppressed (93 vs. 5% above control, E_1 vs. E_1 + 5 μM PD98059), suggesting that the mitogenic action of E_1 is mediated by MAPK

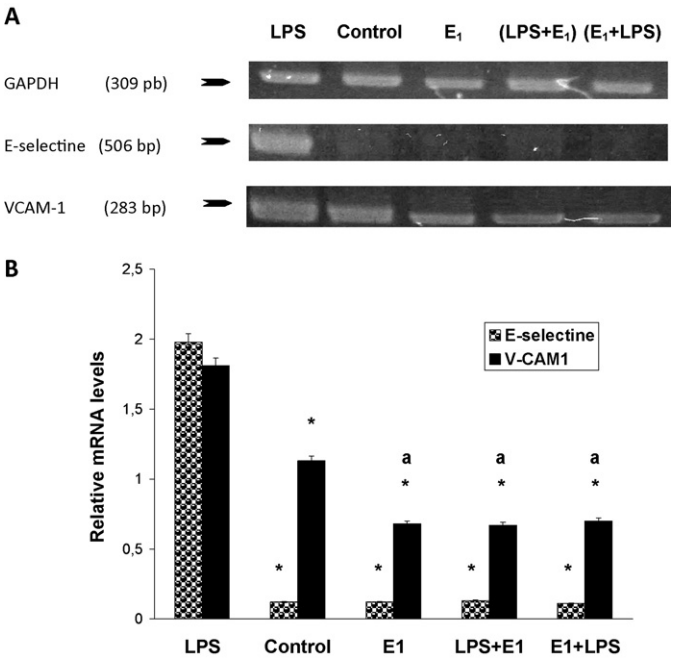

Fig. 4. Regulation by estrone of the mRNA expression of EC adhesion molecules. EC were incubated with 1 μg/ml LPS for 24 h; vehicle alone; 10 nM E_1 for 24 h; 1 μg/ml LPS for 20 h + 10 nM E_1 for additional 5 h; 10 nM E_1 for 5 h + 1 μg/ml LPS for additional 20 h. RT-PCR was performed as described under Section 2. (A) Representative gel photography of E-selectine, VCAM-1, and GAPDH PCR amplification products is shown. The expected band sizes for different molecule products are indicated by arrows. (B) Bars show the relative intensity of each band determined by densitometric analysis (Image J software). Data are presented as E-selectine and VCAM-1 mRNA relative to GADPH mRNA and are the average ± SD of three independent experiments ($n = 4$). *$p < 0.01$ vs. each CAMs level in LPS group; (a) $p < 0.01$ vs. VCAM-1 level in control group.

pathway (Fig. 6). We also tested the role of PKC on the regulation of EC proliferation elicited by E_1. To that end, ^{3}H-thymidine incorporation assays were performed in EC preincubated with the compound Chelerythrine, a PKC inhibitor. Fig. 6 also shows that the presence of the inhibitor did not affect the stimulatory action of the steroid on DNA synthesis (93; 98% above control, E_1; E_1 + chel).

Since EC proliferation is also regulated by estradiol, we evaluated the effect of combined treatment with 17-β-E_2 and E_1. Fig. 7 shows that when each estrogen was added alone, a marked increase

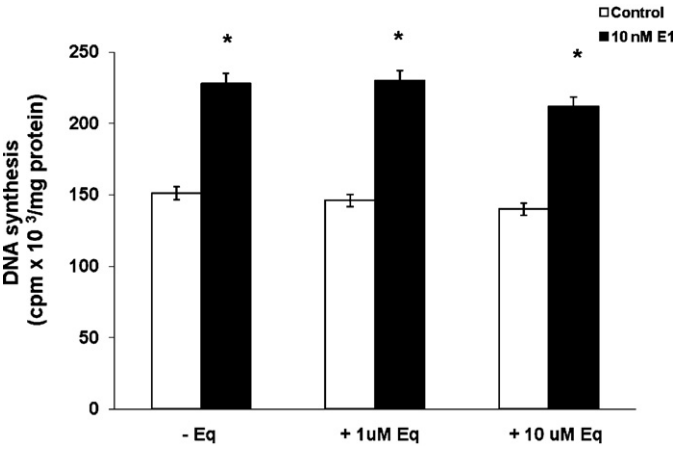

Fig. 5. Effect of Equilin on estrone induced ^{3}H-thymidine incorporation: starved EC cultures were preincubated with 1 or 10 μM Equilin (Eq) for 30 min and then exposed to 10 nM E_1 or vehicle alone for 24 h. ^{3}H-thymidine incorporation was measured as described in Section 2. Results are the average ± SD of four independent experiments ($n = 4$), *$p < 0.001$, respect to each control value.

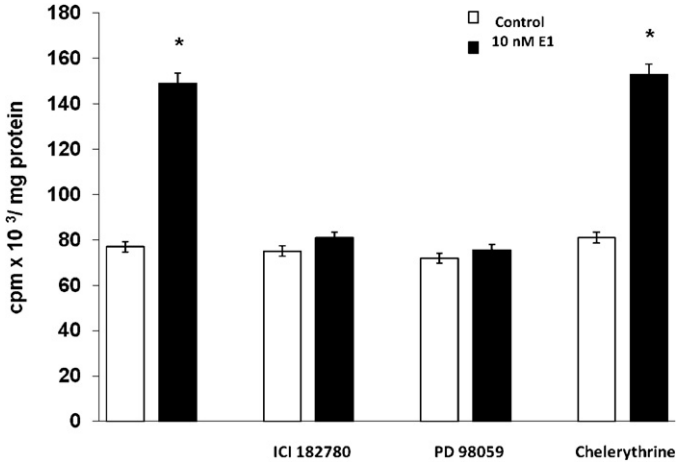

Fig. 6. Effect of ICI 182780, PD98059 and Chelerythrine on the mitogenic action of estrone: starved EC cultures were preincubated with 10 μM ICI 182780, 5 μM PD98059 or 1 μM Chelerythrine and then exposed to 10 nM E_1 or vehicle alone for 24 h. ^{3}H-thymidine incorporation was measured as described in Section 2. Results are the average ± SD of three independent experiments ($n = 4$), *$p < 0.001$, respect to each control value.

in ^{3}H-thymidine incorporation was detected (81; 65% above control 10 nM E_1; 10 nM E_2 respectively), meanwhile the co-treatment with 10 nM E_1 plus 10 nM E_2 blunted the individual steroid action. Indeed no statistically differences were observed between control and 10 nM E_1 + 10 nM E_2 groups. Similar results were observed at 1 nM concentration of each estrogen (Fig. 7). Afterwards we studied non-genomic action of both estrogens on nitric oxide synthesis. To that end, EC were exposed to 5 min treatment with E_1 or 17-β-E_2 added alone or simultaneously. A wide range of concentrations were studied (Table 1). Estrone (1–100 nM) or estradiol (1–100 nM) significantly enhanced NO synthesis (80–106; 72–171% above control, E_1; E_2 respectively). The combination of the two steroids markedly reduced the enhancement in NO production. This inhibitory action was detected in all the ratios of E_1 + E_2 assessed. Although a clear reduction in NO synthesis was detected after all combined treatment, a small incremental increase remained detectable respect to control value (Table 1). Moreover, similar

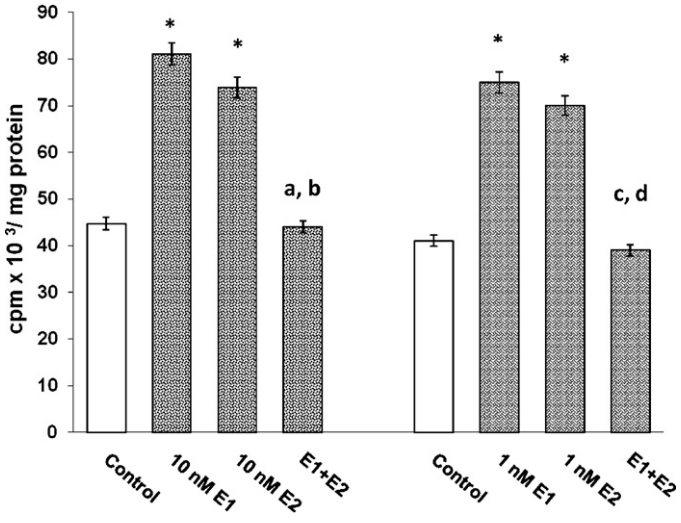

Fig. 7. Effect of E_1 and E_2 co-treatment on EC proliferation: starved EC cultures were exposed to E_1 (1; 10 nM), E_2 (1; 10 nM); 1 nM E_1 + 1 nM E_2; 10 nM E_1 + 10 nM E_2 for 24 h. ^{3}H-thymidine incorporation was measured as described in Section 2. Results are the average ± SD of four independent experiments ($n = 4$), *$p < 0.001$ respect to each control (a) $p < 0.001$ vs. 10 nM E_1; (b) $p < 0.001$ vs. 10 nM E_2; (c) $p < 0.001$ vs. 1 nM E_1; (d) $p < 0.001$ vs. 1 nM E_2.

Table 1
Effect of E_1 and E_2 co-treatment on EC NO production: dose–response study. EC were exposed to E_1, E_2 or $E_1 + E_2$ at different concentrations for 5 min. NO production was measured as described under Section 2. Results are the average ± SD of three independent experiments ($n = 4$).

Treatment	nmol NO/mg protein	% stimulus/above control
Control	0.51 ± 0.03	–
1 nM E_1	0.95 ± 0.05	↑ 87%*
10 nM E_1	1.05 ± 0.07	↑ 106%*
100 nM E_1	0.92 ± 0.08	↑ 81%*
1 nM E_2	0.88 ± 0.07	↑ 72%*
10 nM E_2	1.38 ± 0.08	↑ 171%*
100 nM E_2	1.38 ± 0.09	↑ 170%*
100 nM E_1 + 100 nM E_2	0.71 ± 0.09	↑ 40%**,a,b
10 nM E_1 + 10 nM E_2	0.65 ± 0.04	↑ 27%**,c,d
1 nM E_1 + 1 nM E_2	0.62 ± 0.05	↑ 22%**,e,f
100 nM E_1 + 10 nM E_2	0.63 ± 0.04	↑ 24%**,a,d
1 nM E_1 + 10 nM E_2	0.71 ± 0.08	↑ 40%**,e,d
10 nM E_1 + 1 nM E_2	0.72 ± 0.06	↑ 41%**,c,f
10 nM E_1 + 100 nM E_2	0.73 ± 0.05	↑ 43%**,c,b

[a] $p < 0.01$ vs. 100 nM E_1.
[b] $p < 0.01$ vs. 100 nM E_2.
[c] $p < 0.01$ vs. 10 nM E_1.
[d] $p < 0.01$ vs. 10 nM E_2.
[e] $p < 0.01$ vs. 1 nM E_1.
[f] $p < 0.01$ vs. 1 nM E_2.
* $p < 0.001$ respect to control.
** $p < 0.01$ respect to control.

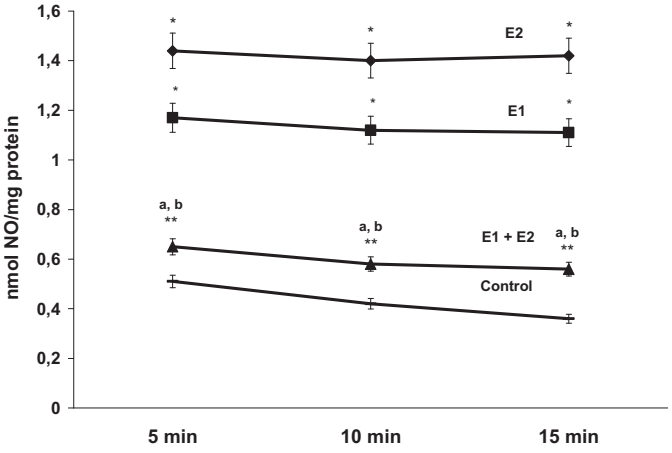

Fig. 8. Effect of E_1 and E_2 co-treatment on EC NO production: time profile. EC were exposed to 10 nM E_1, 10 nM E_2 or 10 nM E_1 + 10 nM E_2 for the time indicated. NO production was measured as described under Section 2. Results are the average ± SD of four independent experiments ($n = 4$), *$p < 0.001$ vs. respective control; **$p < 0.01$ vs. control; (a) $p < 0.01$ vs. 10 nM E_1 at each time treatment; (b) $p < 0.01$ vs. 10 nM E_2 at each time treatment.

impairment in the steroid action was obtained when EC were exposed to the combined treatment for different times (Fig. 8). Again, unlike what was observed in proliferation assays, a small increase in NO production was detected after $E_1 + E_2$ treatment respect to control value.

4. Discussion

In this work we provide evidence that E_1 "per se" modulates endothelial cellular events that play key roles in vascular disease development. The hormone prompted EC growth and survival, and prevented monocyte's adhesion and specific endothelial cells CAMs expression induced by inflammatory conditions. The mitogenic action of estrone on endothelial cells involves the participation of ER and MAPK transduction pathway activation. The presence of estradiol impairs the potential beneficial role of estrone on cell proliferation and vasoactive production.

EC apoptosis is recognized as an important feature in vascular diseases. Atherosclerotic plaques exhibited an enhanced EC turnover with an important increase in apoptosis rates. Moreover, several reports have described that EC programmed death is enhanced through atherosclerosis risk factors such as oxidative stress (Choy et al., 2001; Kockx and Herman, 2000). EC apoptosis would be useful to induce the regression of atherosclerotic lesions, but would also have negative effects on the plaque stability. It has been reported that 17-β-estradiol induces EC survival via inhibition of the apoptotic p38 signaling, but it also promotes vascular smooth muscle cell apoptosis by activating p38 MAPK pathway (Meyer et al., 2009). We showed that physiological concentration of E_1 did not alter basal apoptosis rates, but under oxidative stress conditions the hormone prevents the apoptosis produced by hydrogen peroxide.

One of the earliest events leading to the progression of atherosclerotic disease is leukocyte recruitment and transendothelization after injury. EC activation by proinflammatory cytokines or bacterial endotoxins predisposes to leucocytes recruitment. The presence of adhesion molecules on EC surface is crucial for this cellular interaction. E-selectin is an inducible endothelial CAM involved in tethering and transition to stable arrest. VCAM-1 triggers monocytes adherence and transendothelial migration to the intima (Jung and Ley, 1999; Rao et al., 2007). In the present study, we showed that E_1 treatment not only reduced monocytes adhesion under basal conditions but also attenuated bacterial LPS-induced monocytes adhesion. It has been reported that in vein endothelial cells the proinflammatory agent LPS increased transcription of adhesion molecules target genes (Cronstein et al., 1992; Osborn et al., 1989). We confirmed that in our experimental model, LPS also induced monocyte's adhesion associated with an up regulation of specific CAMs mRNA expression. We obtained evidence that E_1 does not affect the synthesis of mRNA of E-selectin and VCAM-1, but prevents the LPS-induced increase. This down regulation was detected when the steroid was added either before or after LPS stimulation. These results would suggest a vasoprotective role of estrone against vascular inflammation.

It is unknown if E_1 has a specific receptor or acts via the ER. Evidence in the literature reported that E_1 exhibits a significative binding affinity to ERα, (Kuiper et al., 1997; Gruber et al., 2002). Moreover, Sasson and Notides (1983) showed that in calf uterine ER, E_1 occupies the same site that E_2 does. Using rat aortic strips or vascular smooth muscle cells we have shown that either rapid or long term effects mediated by estrone were suppressed by the high affinity ER antagonist, ICI182780 (Massheimer et al., 2006; Rauschemberger et al., 2008). In agreement with these observations, the data provided in Fig. 6 suggests that ER would be involved in E_1 mitogenic action on EC.

It is widely considered that steroid hormones act via their classical mechanism of action regulating target genes transcription after binding nuclear receptors (Truss and Beato, 1993). However, the existence of steroid non-genomic actions has been widely accepted during the last decade (Wehling and Lösel, 2006). Nowadays, a two step model of steroid effects that integrates genomic and non-genomic actions has been proposed. This model was originally developed for aldosterone (Chambliss et al., 2000) and later expanded to others steroids (Lösel et al., 2003; Lösel and Wehling, 2003). In vascular endothelial cells, gene transcription and cell biological effects of estrogens emanate from rapid and specific signaling, integrating cell surface and nuclear action (Pedram et al., 2002). MAPK cascade is among the most common and important signaling pathways activated by both receptor and non-receptor tyrosine kinase. This intracellular network controls diverse cellular functions including proliferation and migration. ERK pathway

activation has been associated with rapid signaling elicited by estrogen receptor that leads to downstream transcription factors phosphorylation which then regulates gene transcription (Fu et al., 2007; Simoncini, 2009). We had previously demonstrated that E_1 induced a rapid stimulation of MAPK activity (Rauschemberger et al., 2008). In this work, we found that, the increase in EC proliferation mediated by E_1 was suppressed by the MEK inhibitor PD98059 suggesting that E_1 mitogenic action also depends on steroid MAPK activation. On the other hand, although the PLC/PKC transduction system is suitable to be activated by E_1 (Massheimer et al., 2006; Rauschemberger et al., 2008), this cascade does not mediate the hormonal regulation of EC growth.

Unidirectional conversion of E_1 to E_2 is catalyzed by 17-β-HSD 1, an enzyme that could be blocked by Equilin compound through its binding to its catalytic subunit (Sawicki et al., 1999; Rauschemberger et al., 2008). The presence of 17-β-HSD 1 in endothelial cells has been reported (Murakami et al., 1999). When EC were preincubated with Equilin the mitogenic action of estrone was not altered. These results suggest that this stimulation of DNA synthesis induced by E_1 could not be attributed to its conversion to E_2.

Estradiol and estrone are concomitantly present in the blood stream of both fertile and postmenopausal women. In climacteric women subjected to hormonal replacement therapy (HRT), the major estrogen provided by the conjugated equine estrogens is estrone (Notelovitz, 2006). Cellular and molecular actions of both estrogens at vascular level exhibit distinct features (Kappert et al., 2006; Rauschemberger et al., 2008). Here we showed that when co-treatments were performed, the mitogenic action of each individual steroid was suppressed. Moreover, this interference was also detected in nitric oxide synthesis stimulation, a non-genomic action undertaken by each estrogen. It is known that in fertile women estradiol serum levels are higher than estrone, while in postmenopausal women this ratio is reversed ($E_1 > E_2$). In the combined treatment experiments we checked the effect of different dose relationships of both estrogens, $E_1 > E2$ (10 nM E_1 + 1 nM E_2; 100 nM E_1 + 10 nM E_2) or $E_2 > E_1$ (10 nM E_2 + 1 nM E_1; 100 nM E_2 + 10 nM E_1). Interference in the hormonal response was observed in all dose ratios assayed. These results reveal the existence of hormonal interactions between both ovarian estrogens. We have previously reported that estrone mechanism of action at vascular level involves NOS; COX; PLC/PKC transduction pathways activation (Sellés et al., 2005; Massheimer et al., 2006). Perhaps, the alterations observed in co-treatment assays with respect to each individual estrogen biochemical action, would be attributed to a cross talk among different transduction pathways activated by each steroid. This topic would be focused in future studies.

Although the direct effects of E_1 on EC reported here (stimulation of EC proliferation, inhibition of monocyte adhesion and EC apoptosis) would be considered beneficial for vascular homeostasis, we had previously demonstrated that the steroid also has certain adverse effects such as a marked increase in VSMC proliferation, stimulation of the synthesis of the vasoconstrictor and proaggregant agent thromboxane (Sellés et al., 2005; Rauschemberger et al., 2008). Using in vivo assays, we reported that in rats deprived of ovarian activity E_1 was unable to activate NOS cascade, and this stimulatory action was restored after injecting estradiol to the ovarectomized rats. Taking all this evidence together leads us to speculate that the biochemical action of estrone in the vascular system may be the sum of desirable and undesirable actions. Our results belong mainly to isolated cells. Since, vascular health depends on the integration of cellular responses from blood cells (leukocytes, platelets), endothelial cells, and muscle cells; at present it is difficult to draw a parallel between our results and the in vivo situation.

5. Conclusions

In summary we provided evidence that the natural estrogen estrone exhibits a remarkable biological effect at vascular level. The steroid displays an own mechanism of action with distinctive features that leads to cellular behaviour regulation either under basal or injury conditions. Further investigations are required for a better understanding of the physiological relevance of our findings.

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