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ScienceDirect
Life Sciences 82 (2008) 115–123
Life Sciences
www.elsevier.com/locate/lifescie

The direct action of estrone on vascular tissue involves genomic and non-genomic actions

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Received 17 July 2007; accepted 22 October 2007

Abstract

A two step model mechanism of steroid action has been recently postulated. In this study, we test the hypothesis that, the biochemical action of estrone (E_1) on vascular tissue could be performed via genomic and non-genomic actions. Rat aortic rings or vascular smooth muscle cell cultures (VSMC) were used to test the effect of the hormone on nitric oxide (NO) production, protein kinases activities and cell proliferation. Our data showed that estrone increased NO synthesis between 30 s and 20 min treatment, and this stimulatory effect was dependent on MAPK cascade activation, since it was prevented in the presence of a MAPK inhibitor (PD98059). Using a phosphorylation assay, we also showed that E_1 significantly increased MAPK activity. The effect of the hormone on PKC activity was measured in concentrations and time course studies. Direct treatment of rat aortic homogenates with E_1 significantly enhanced PKC activity (1–10 fold increase, $p<0.01$) at all concentrations (1; 10; 50 nM) and time tested (1–10 min). We demonstrated that 24 h of E_1 treatment markedly increased VSMC proliferation (53% above control), and this effect was suppressed by a PKC inhibitor. The rapid and the long term effects of the hormone were completely suppressed in the presence of an estradiol receptor antagonist (ICI 182780). In summary, we provided evidence that, the steroid exerts both non-genomic and genomic actions, the former associated with MAPK kinase dependent on NO production, and the latter related with induction of VSMC proliferation involving PKC pathway activation.

Keywords: Estrone; Vascular smooth muscle cells; Nitric oxide; Cell proliferation

Introduction

The mechanism of action of steroids hormones involves genomic and non-genomic pathways. In the last few years, a two step model mechanism of steroid action has been postulated (Falkenstein et al., 2000). Steroid rapid action at membrane level triggers intracellular transduction pathway activation that would converge in the synthesis of transcriptional factors required for the genomic action (Haynes et al., 2002). An example on this is the effect of 17-β-estradiol (E_2) on vascular tissue regulating vasomotor tone (Chambliss and Shaul, 2002) and attenuating atherosclerotic lesions (Mendelsohn and Karas, 1999).

Vascular smooth muscle cell (VSMC) migration and proliferation are main events responsible of the progression of sclerotic lesion. VSMC exist in the normal blood vessel wall in a quiescent differentiated state with low rate of cell proliferation and turnover. Inflammatory or mechanical injury to the artery wall causes VSMC dedifferentiation, lose contractile properties and induces a proliferative and often motile phenotype (Ross, 1993; Pauly et al., 1994). It has been proposed that subpopulations of smooth muscle cells exist in the arteries of various species, including human (Hao et al., 2003). The study of the biological features and the regulation of this VSMC heterogeneity should be useful for the better understanding of their role in physiologic and pathologic situations.

Nitric oxide (NO) is a potent vasodilator synthesized by nitric oxide synthase (NOS) through the conversion of L-arginine to L-

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0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.10.020

citrulline (Palmer et al., 1987). NO prevents platelet aggregation (Ignarro, 1989) and neutrophil adhesion to the endothelium (Tsao et al., 1995). Alterations in NO synthesis can induce the development of vascular disease such as hypertension and atherosclerosis (Mendelsohn and Karas, 1999).

Cardiovascular disease is the leading cause of death in the Western world (Braunwald, 1997). The incidence of cardiovascular disease in premenopausal women is lower than in men, but it reaches similar value after menopause. These findings are attributed to cardioprotective effect of females sex hormones, particularly estrogens (Kannel and Wilson, 1995).

Numerous studies showed that E_2 exerts vasodilator, anti-inflammatory and antiatherosclerotic properties, as well as, favourable effects on lipid profile (Clarkson et al., 2001; Haarbo and Christiansen, 1996). In vitro studies demonstrated that E_2 maintains endothelial integrity, prevents leukocyte adhesion, inhibits interleukin expression and reduces neointima formation (Mendelsohn and Karas, 1999). However, a series of randomised, placebo-controlled trials of hormone therapy, in both primary and secondary prevention, failed to confirm cardiovascular benefit of estrogens (Ouyang et al., 2006). It is important to note that most of the experimental studies were carried out using E_2, but little information is yet available about the possible effects of other estrogens or estrogen metabolites.

Estrone (E_1) represents the major remaining estrogen in circulation beyond menopause. Peripheral aromatization of androstenedione and testosterone is the main source of circulating E_1 (Bulun et al., 1999; Grow, 2002). Aromatase is the enzyme involved in this conversion which has been found in different tissues including VSMC (Harada et al., 1999). It has been proposed that estriol (E_3) and E_1 sulfate similarly to E_2 might have atheroprotective effects, since they inhibit the production of mRNAs of cytokines (IL-1 and IL-6) and growth regulators (PDGF) that induced VSMC multiplication in the early initiation of atherosclerosis (Kikuchi et al., 2000). However, their direct effects on VSMC proliferation and migration have not been tested.

We have previously shown that, in rat aortic tissue, E_1 induced a rapid non-genomic stimulation of NOS and COX activity, and inhibited platelet aggregation in a NO dependent manner (Sellés et al., 2005). Furthermore we recently demonstrated that E_1 elicits its non-genomic action through the activation of PLC/DAG transduction pathway, and cross talk between NOS and COX systems, or PLC and PI3K pathways (Massheimer et al., 2006). In this study, we test the hypothesis whether, the biochemical action of E_1 on vascular tissue would be performed via a two step mechanism of action involving genomic and non-genomic actions.

Materials and methods

Materials

Estrone and PD98059 were obtained from Sigma Chemical Co. (St. Louis, USA). ICI 182780 was purchased from Tocris Bioscience (Park Ellisville, USA). Genistein, chelerythrine and calyculin A were obtained from Alomone Labs (Jerusalem, Israel), Griess reaction solutions 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 were obtained from PAA Laboratories (Pasching, Austria). Dulbecco's modified Eagle's medium modified and all other reagents were purchased from Sigma Chemical Co. (St. Louis, USA).

Animals

Sexually mature female Wistar rats (6 month old) that had progressed through at least 3 consecutive estrous cycles were fed with standard rat food, given water ad libitum and maintained on a 12 h light 12 h dark cycle. The estrous cycle was evaluated by optical microscope examination of vaginal smears, and the animals chosen for the experimental design were those with similar cycle length and the same number of days spent at each stage of the cycle.

Rat aortic strips (RAS) preparations

Animals were killed by cervical dislocation; the thoracic aorta was immediately removed and placed in cold DPBS solution, cleaned of adherent connective tissue, and cut in 1.5 cm strips. Special care was taken to preserve the integrity of the endothelial layer. The strips were placed in the incubation medium (in mM): 125.4 NaCl; 5.9 KCl; 1.2 $MgCl_2$; 1.5 $CaCl_2$; 11.5 glucose; 10 Hepes pH: 7.35 and preequilibrated at 37 °C in a water thermostatized shaking bath (Mendiberri et al., 2006).

Rat aortic homogenates (RAH) preparations

Animals were killed by cervical dislocation, the thoracic aorta was immediately removed and placed in cold DPBS solution, cleaned of adherent connective tissue, and homogenized in adequate buffer for enzymatic assays: *for PKC assay* (in mM): 20 Tris–HCl, pH 7.4; 330 sucrose; 1 EGTA; 20 NaF; 1 DTT and 20 μg/mL aprotinine; *for MAPK assay* (in mM): 20 Tris–HCl pH 7.4; 50 NaF; 1 EGTA; 270 sucrose; 1 EDTA; 1 DTT; 1 sodium orthovanadate; 2 μg/mL pepstatin A and 2 μg/mL aprotinine.

Vascular smooth muscle cells cultures

Vascular smooth muscle cells (VSMC) were obtained from aortic ring explants. To that end 4–6 weeks old Wistar female rats were employed (Yeh et al., 2002). The full length of thoracic aorta was aseptically removed and cut into ring segments (1–1.5 mm). Ring explants were seeded in the matrix-coated petri-dishes (NUNC) containing 90% phenol red free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum; 3.7 mg sodium bicarbonate, 100 U/mL penicillin, 1 μg/mL streptomycin, 2 mM glutamine and 2.5 μg/mL amphotericin B (medium A). Explants were cultured at 37 °C in 95% air/5% CO_2 atmosphere for 5–6 days (step 1), removed from the dishes, and transferred into new culture dishes containing medium A. After another 5 days (step 2), the aortic rings were transferred again to new matrix-coated petri-dishes (NUNC) containing medium A and cultured for 5 additional days in order to obtain pure populations of smooth muscle cells. The rings were discarded,

and the remaining cells were allowed to reach confluence (step 3). Only step 3 cultures were used for all studies. The cells were seeded in 12 well plates and grown to confluence to perform the experiments. The identity of the VSMC was determined by the positive inmunocytochemistry reactivity to smooth muscle specific alpha-actin, using DakoCytomation EnVision system.

Measurement of NO production by Griess method

Aortic strips were preincubated in (in mM): 145 NaCl; 5.0 KCl; 1.2 $MgSO_4$; 1.0 $CaCl_2$; 10 glucose; 10 Hepes pH: 7.35, for 15 min at 37 °C, and then exposed to E_1 treatment for different time intervals (30 s–20 min). Respective control group with vehicle alone (ethanol <0.1%) was also processed. When antagonist or inhibitors compounds were used, they were added to the incubation medium 30 min prior hormonal treatment. Aortic tissue was dissolved in 1 N NaOH, and protein determination was performed by Lowry reaction. NO production was determined by Griess reaction using a Hitachi U-1000 spectrophotometer (Griess, 1879). Results were expressed in nmol NO/mg protein.

Assay of PKC activity

PKC activity was determined by the incorporation of ^{32}P from [$\gamma^{32}P$]-ATP into histone in the presence or absence of $CaCl_2$, phosphatidylserine and DAG (Massheimer et al., 2006). The assay buffer employed was: 0.2 M Tris–HCl, pH 7.4, 0.1 M $MgCl_2$, 0.1 M EGTA, 12.5 mM NaF, 1 mM DTT, 20 μg/mL aprotinine plus 5 μg histone (type III-S) with or without 1 mM $CaCl_2$, 10 μg phosphatidylserine and 0.75 μg DAG. 100 μg of RAH was incubated with different concentrations of E_1 for 5 min. Immediately after ATP-ATP-γ-^{32}P (100 μM; 0.2 μCi) were added for 5 additional minutes. The reaction was stopped by transferring an aliquot of assay mixture into Whatmann P-81 disk, washed, dried, and radioactivity quantified by liquid scintillation. Results were expressed in pmol of Pi/min per mg protein.

Assay of MAPK activity

Rat aortic homogenates were immunoprecipitated with anti-MAPK kinase (p42 and p44), extensively washed, and an aliquot was suspended in 100 μL assay buffer: 25 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 20 μg/mL aprotinine, 20 μg/mL pepstatin, 1 mM DTT, 1 μM okadaic acid, plus 20 μg/assay Basic Myelin as an exogenous substrate for MAP kinase. Treatment was done by incubation for 5 min with 10 nM E_1. Immediately after ATP-ATP-γ-^{32}P (100 μM; 0.2 μCi) were added for 5 additional minutes. The reaction was stopped by transferring an aliquot of assay mixture into Whatmann P-81 disk, washed, dried, and radioactivity quantified by liquid scintillation. Results were expressed in pmol of Pi/min per mg protein (Buitrago et al., 2003).

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

Subconfluent VSMC that were cultured in 12-well culture plates were made quiescent by replacing the culture medium by serum-free DMEM for 1 day. The media was changed to DMEM plus 1% serum and the monolayers were exposed to E_1 (10 nM) for 24 h. During the last hour of treatment the cells were pulsed with 1 μCi/mL of [^{3}H]-thymidine. When the compounds ICI 182780 or chelerythrine 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 N. Radioactivity was measured by liquid scintillation, and [^{3}H]-thymidine incorporation per well was normalized to protein content (Kyaw et al., 2002).

Vascular smooth muscular cells migration assay

Vascular smooth muscular cells (3×10^5) were seeded in 60-mm NUNC dishes with DMEM containing 10% FBS and grown to confluence. The cells were starved for 12 h with serum-free medium, wounded by pressing a razor blade down on the dish to cut the cell layer. The blade was then gently moved to one side to remove part of the monolayer. Immediately, the cells were washed twice with PBS and cultured in fresh DMEM containing 1% FBS plus E_1 or vehicle control (isopropanol <0.1%). After 24 h of culture, the cells were fixed in glutaraldehyde 0.1% and stained with hematoxylin–eosin. Migration was quantified by counting the number of cell nuclei that crossed the line demarcated in at least seven different microscopic fields (×100) representative of each culture plate. Results are expressed as means±SEM of number of cells/field (Burk, 1973; Pedram et al., 2002). Each experiment was repeated at least three times.

Measurement of estradiol concentration

Estradiol concentration was measured in RAS incubation medium after 10 min treatment with 10 nM E_1, using

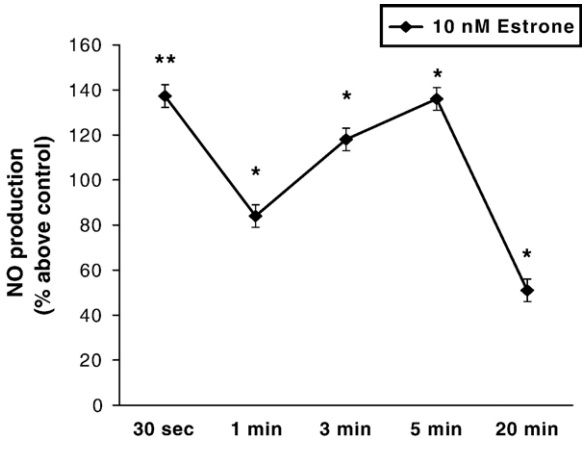

Fig. 1. Effect of E_1 on NO production: time course profile: RAS were exposed to E_1 at the time indicated. Control to each time point was also performed. NO production was measured by Griess method as described in Materials and methods section. Results are expressed as % above each control, and represent the average±SD of three independent experiments ($n=4$), *$p<0.01$, **$p<0.05$, with respect to control value: 0.44±0.021 nmol NO/mg protein.

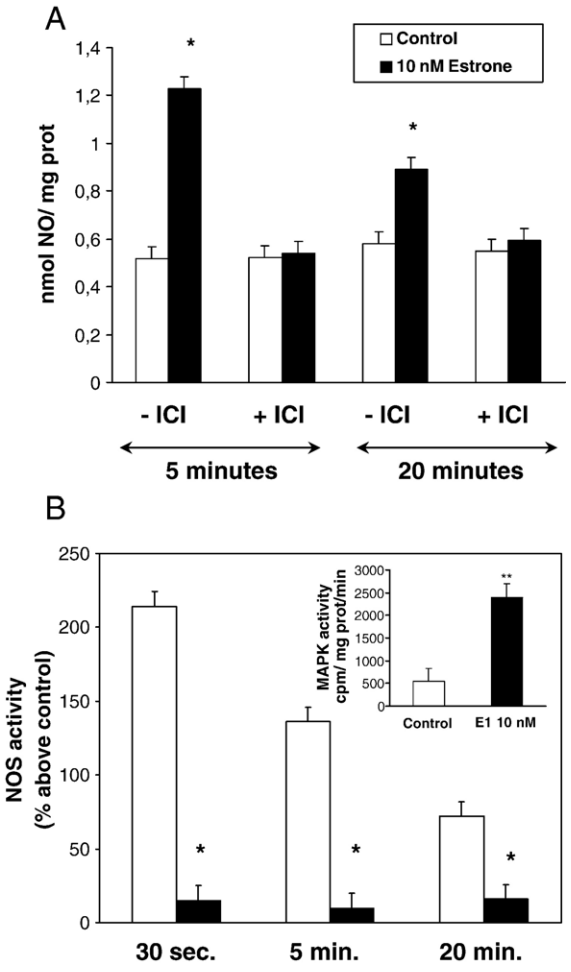

Fig. 2. Effect of ICI 182780 and PD98059 on E_1-NO synthesis: A) RAS were incubated in absence or presence 1 μM ICI 182780 and then exposed to 10 nM E_1 at the time indicated. B) RAS were incubated in absence (white bars) or presence (black bars) of 10 μM PD98059 and then exposed to 10 nM E_1 at the time indicated. NO production was measured by Griess reaction as described under Materials and methods. Results are the average±SD of three independent experiments ($n=4$), *$p<0.01$. *Insert*: Aortic homogenates were exposed to 10 nM E_1 for 1 min. MAPK activity was measured as described under Materials and methods. Results are the average±SD of three independent experiments ($n=4$), **$p<0.02$.

a radioimmunoassay. The commercially available kit exhibits a 100% assay reactivity with 17-beta estradiol, and 1.1% of cross reactivity with estrone (Diagnostic Products Corporation).

Table 1
Effect of calyculin A on the stimulation of NO production induced by E_1

Treatment	nmol NO/mg protein	
	−10 nM calyculin	+10 nM calyculin
Control	0.26 (±0.06)	0.34 (±0.04)
10 nM E_1	0.44 (±0.028)[a]	0.75 (±0.002)[b; c]

RAS were incubated in absence or presence 10 nM calyculin A (Cal) and then exposed to 10 nM E_1 for 5 min. NO production was measured by Griess method as described in Materials and methods section. Results are the average±SD of three independent experiments ($n=4$), [a]$p<0.05$, [b]$p<0.01$ with respect to control value; [c]$p<0.001$ E_1+Cal vs E_1−Cal.

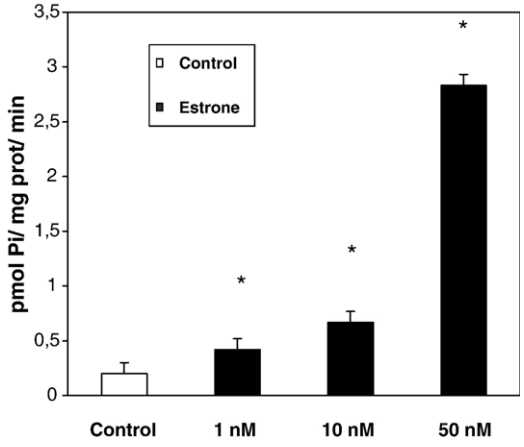

Fig. 3. Effect of E_1 on PKC activity: Aortic homogenates were exposed to different E_1 concentrations for 3 min. PKC activity was measured as described in Materials and methods section. Results are the average±SD of three independent experiments ($n=4$), *$p<0.001$.

Statistical analysis

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

Results

First we evaluated the time course of the hormone action on NO synthesis. To perform these experiments, an E_1 concentration of 10 nM was selected from our previous studies of E_1 NOS stimulation (Sellés et al., 2005). As shown in Fig. 1, E_1 significantly increased NO production at all incubation times

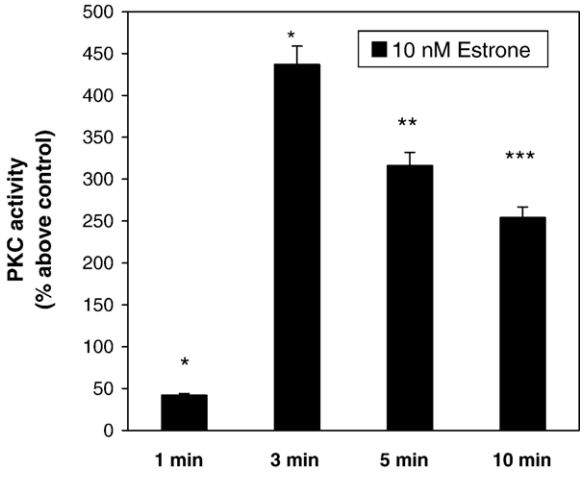

Fig. 4. Effect of E_1 on PKC activity: time course profile. Aortic homogenates were exposed to 10 nM E_1 concentrations at indicated times. PKC activity was measured as described in Materials and methods section. Results are the average±SD of three independent experiments ($n=4$), *$p<0.05$, **$p<0.01$, ***$p<0.001$.

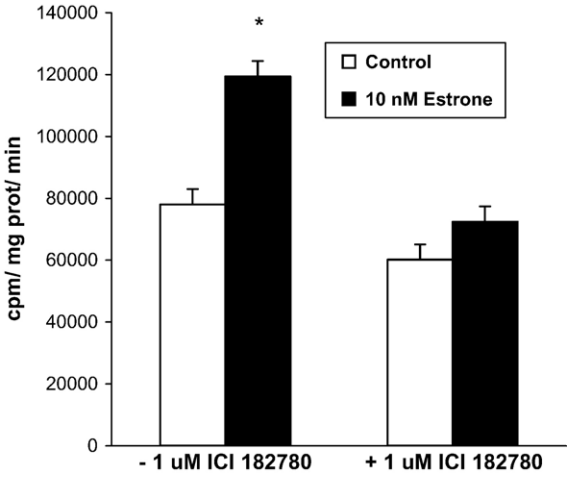

Fig. 5. Regulation of VSMC proliferation by estrone: effect of ICI 182780. Cultured VSMC in 1% FBS were preincubated in absence or presence 1 μM ICI 182780 and then exposed to 10 nM E_1 for 24 h. ^{3}H-Thymidine incorporation was measured as described in Materials and methods section. Results are the average±SD of three independent experiments ($n=4$), *$p<0.01$.

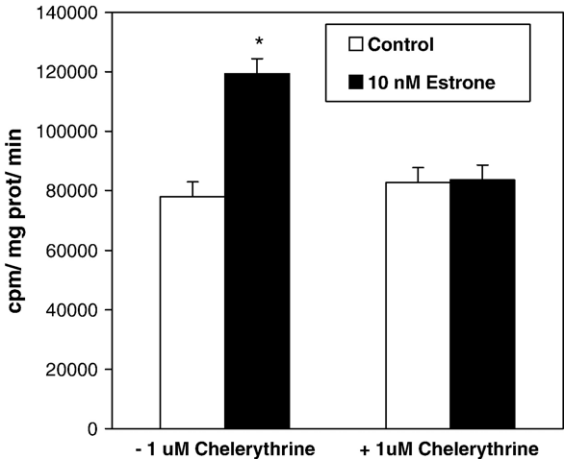

Fig. 6. Effect of chelerythrine on estrone induced ^{3}H-Thymidine incorporation: VSMC cultured in FBS 1% were preincubated in absence or presence 1 μM chelerythrine and then exposed to 10 nM E_1 for 24 h. ^{3}H-Thymidine incorporation was measured as described in Materials and methods section. Results are the average±SD of three independent experiments ($n=4$), *$p<0.01$.

(137 to 51% above each control, $p<0.01$). We tested the possibility whether the estradiol receptor (ER) would participate in E_1 NO production. For this purpose we used the compound ICI 182780, a high affinity estrogen receptor antagonist. The presence of ICI 182780 suppressed the increase in NO production induced by 5 min treatment with 10 nM E_1 (0.52±0.09 vs 1.23±0.04; 0.52±0.03 vs 0.54±0.007; nmol NO/mg protein, Control vs 10 nM E_1, in the absence or presence of ICI 182780 respectively). This effect was also observed after 20 min treatment (Fig. 2A). As a positive control of the specificity of the antagonist we measured the effect of ICI 182780 on E_2 NO production. We have previously demonstrated that in our experimental system, E_2 induced a very fast stimulation of NOS activity (Sellés et al., 2001). The addition of ICI 182780 completely blocked the rise in NO evoked by E_2 (data not shown).

To asses the participation of MAPK pathway in E_1 signal transduction, we studied the effect of this steroid on MAPK activity. As shown in Fig. 2B (insert), a significant stimulus of MAPK activity was observed after the incubation of RAH with 10 nM E_1 (341% above control, $p<0.02$). In order to check the role of MAPK in the stimulation of NO production, the compound PD98059, a specific inhibitor of MAPK cascade was employed. RAS were preincubated for 30 min with 1 μM PD98059 and immediately exposed to 10 nM E_1. Fig. 2B shows that the stimulation of NO production induced by the hormonal treatment was prevented by PD98059. Since cellular signalling involves either phosphorylation–dephosphorylation mechanisms, we evaluated the effects of phosphatases on the NO production elicited by E_1. We used the potent inhibitor of phosphatases PP-1 and PP-2, the compound calyculin A. RAS were preincubated with 10 nM calyculin and exposed to E_1 for additional 5 min. As shown in Table 1, the increase in NO production induced by the hormone in the presence of calyculin, was significantly higher than in the absence of the inhibitor (120 vs 66% above control, respectively, $p<0.001$). These results would suggest that, the effect of estrone on NO production could be due to the activation of both kinase and phosphatase pathways.

Having in mind that we have previously shown (Massheimer et al., 2006) that E_1 enhanced DAG formation by simulation of PLC activity, we measured the effect of the hormone on PKC activity. In Fig. 3, it can be observed that, direct treatment of RAH with physiological concentrations of E_1 (1; 10; 50 nM) significantly enhanced PKC activity (110; 235; 1315% above

Table 2
Effect of cycloheximide and actinomycin D on ^{3}H-thymidine incorporation elicited by estrone

Treatment (24 h)	^{3}H-Thymidine incorporation (cpm 10^3/mg protein)		
		+100 μM cycloheximide	+10 μg/mL actinomycin D
Control	264±35	184±20	79±26
10 nM E_1	401±75*	193±23	80±13

VSMC cultured in FBS 1% were incubated with 10 nM E_1 for 24 h. Cycloheximide (100 μM) or actinomycin D (10 μg/mL) were added to the incubation medium 30 min prior hormonal treatment. ^{3}H-Thymidine incorporation was measured as described under Materials and methods. Results are the average±SD of three independent experiments ($n=4$), *$p<0.001$.

Table 3
Effect of PD98059 on the stimulation of ^{3}H-thymidine incorporation induced by TPA or E_1

Treatment	cpm 10^3/mg protein	
	−1 uM PD98059	+1 uM PD98059
Control	228 (±19)	176 (±20)
100 nM TPA	494 (±74)*	334 (±33)*
10 nM E_1	374 (±75)**	285 (±28)*

VSMC cultured in FBS 1% were preincubated for 30 min in absence or presence 1 μM PD98059 and then exposed to 10 nM E_1 or 100 nM TPA for 24 h. ^{3}H-Thymidine incorporation was measured as described in Materials and methods section. Results are the average±SD of three independent experiments ($n=4$), *$p<0.001$; **$p<0.01$.

Table 4
Effect of equilin on estrone induced NO production and ^{3}H-thymidine incorporation

Treatment	NO production (nmol NO/mg protein)		^{3}H-Thymidine incorporation (cpm 10^3/mg protein)	
	−Equilin	+Equilin	−Equilin	+Equilin
Control	0.82±0.05	0.78±0.03	135±8.1	128±13
10 nM E_1	1.28±0.04*	1.28±0.04*	258±33*	252±30*

Nitric oxide production: RAS were exposed to 10 nM E_1 for 10 min and NO production was measured by Griess reaction as described under Materials and methods. *^{3}H-Thymidine incorporation*: VSMC cultured in FBS 1% were incubated with 10 nM E_1 for 24 h and ^{3}H-thymidine incorporation was measured as described under Materials and methods. In both experimental assays equilin (1 μM) was added to the incubation medium 30 min prior hormonal treatment. Results are the average±SD of three independent experiments (n=4), *p<0.001.

control respectively). The time course study shows that the stimulatory action of E_1 on PKC activity was already seen at 1 min treatment (42% above control), followed by a pronounced peak at 3 min (437% above control), and then the response declined towards 10 min (Fig. 4).

The aortic rings represent an experimental system in which coexist endothelial and smooth muscle cells. In order to investigate the individual effect of E_1 on these different cells types, we developed VSMC cultures isolated from rat aortic explants. Using ^{3}H-thymidine incorporation we studied the genomic action of E_1 on the regulation of cell proliferation. We first checked the time course of ^{3}H-thymidine incorporation under basal conditions (8 to 96 h of culture with 1% FBS). The maximal incorporation was detected between 24 and 36 h (data not shown). Then we studied the effect of E_1 on DNA synthesis. As shown in Fig. 5, the hormone markedly increased VSMC proliferation after 24 h of treatment (53% above control, p<0.01). The genomic nature of this steroid action was demonstrated by the suppression of the proliferative effect by gene transcription (actinomycin D) or protein synthesis (cycloheximide) inhibitors (Table 2). To asses whether ER would be involved in this mitogenic effect, DNA synthesis was measured in the presence of 1 μM ICI 182780. The pure antiestrogen effectively blocked the ability of 10 μM E_1 to stimulate ^{3}H-thymidine incorporation (Fig. 5). Subsequently, we studied whether the mitogenic effect of estrone was dependent on PKC pathway. VSMC proliferation was measured after 24 h treatment with the phorbol esther TPA, a direct PKC activator (Table 3). TPA markedly increased ^{3}H-thymidine incorporation (116% above control p<0.001). Then, we measured the effect of E_1 on VSMC proliferation in the

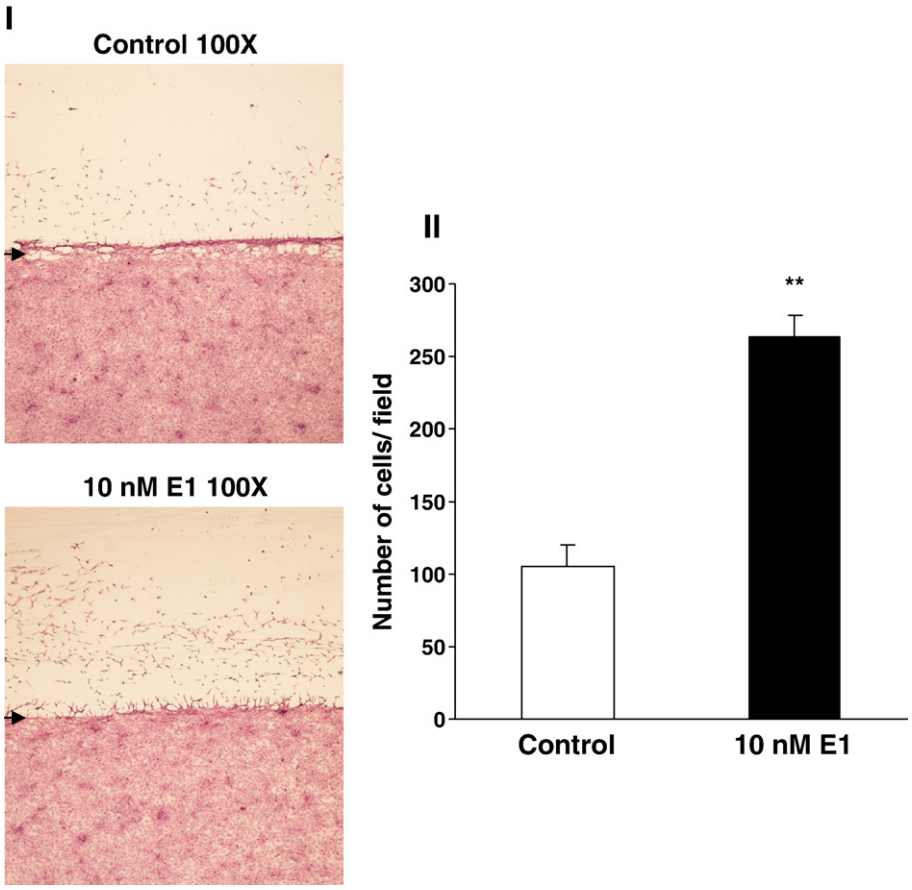

Fig. 7. E_1 effect on vascular smooth muscular cells migration. Confluent VSMC cultured on 60-mm NUNC dishes were serum starved for 12 h. After scraping the cells besides the wound (indicated by the arrow), the half remaining monolayer was treated with 10 nM E_1 for another 24 h and then processed as described in Materials and methods. I) Images captured after stained with hematoxylin–eosin. II) Bars represent the means±SD of the average number of migrating cells measured in three separated experiments performed by quadruplicate. **p<0.01.

presence of a PKC inhibitor (chelerythrine compound). Under this condition, the steroid was unable to stimulate DNA synthesis (Fig. 6), whereas the inhibitor alone had no effect compared to control values. These results suggested that the mitogenic response of E_1 depends on the activation of PKC pathway. Moreover, we studied whether VSMC proliferation involved a cross talk between PKC and MAPK pathways. Table 3 shows that, the presence of the MAPK inhibitor (PD98059) did not modified the mitogenic action of TPA or E_1, excluding a probable interaction between both transduction systems.

Since estrone would be converted in estradiol through the catalytic activity of 17-beta-hydroxysteroid dehydrogenase type 1 (17HSD-1) (Puranen et al., 1997), we measured the effect of E_1 on NO production and VSMC proliferation after 17HSD inhibition. To that end, we selected the compound equilin (3-hydroxyestra1,3,5,7 tetraen-17-one), a specific inhibitor of 17HSD-1 (Sawicki et al., 1999). RAS or VSMC were preincubated for 30 min with equilin compound, and immediately after estrone was added for 10 min or 24 h respectively. Table 4 shows that even though the conversion of E_1 to E_2 was blocked, estrone maintained its ability to increase NO production or to stimulate cell proliferation (56 vs 64; 91 vs 97% above control each control value, E_1 vs E_1+equilin respectively). In order to confirm that E_1 to E_2 conversion was not required for estrone effects, we measured estradiol concentration in RAS incubation medium, after 10 min treatment with E_1. No significant differences between control and treated group were observed (1.01 ± 0.09 vs 0.98 ± 0.10 pg/mL, control vs estrone).

Finally, we evaluated the effect of E_1 on VSMC migration. Fig. 7 shows photographs of a representative assay. There can be observed the cells that crossed the line demarcated and migrated to the denuded area after 24 h treatment with 10 nM E_1 or vehicle alone (control). After the cells were fixed and stained, cells nuclei number per field were counted. The data provide evidence that E_1 stimulated cell migration, inducing a 2.5 fold enhancement in VSMC movement (105 ± 9 vs 264 ± 22; number of cells/field, control vs E_1; $p < 0.01$).

Discussion

The results presented in this study provide evidence that the mechanism of action of E_1 on vascular tissue implicates genomic and non-genomic actions. The steroid rapidly increased MAPK activity and this effect was involved in the acute stimulation of NO production. On the other hand, the long term effect of E_1 promoting VSMC proliferation was associated with PKC transduction system, a second messenger cascade that was non-genomically activated by E_1. We found that either the rapid or the long term effects elicited by the hormone were blunted by the ER antagonist ICI 182780.

It is known that estrone would be converted in estradiol through the catalytic activity of 17-beta-hydroxysteroid dehydrogenase type (17βHSD), a protein family composed by thirteen members. Undirectional conversion of E_1 to E_2 is catalyzed by types 1; 7; 12. Expression of 17βHSD-7 in vascular tissue remains unclear. Although type 12, the most recent member reported, is ubiquitously expressed in liver, muscle and kidney, there is no clear information about substrate specificities (Mindnich et al., 2004; Song et al., 2006). We supposed that, in our experimental system, the E_1 to E_2 conversion catalyzed by types 7 and 12 would be improbable. The 17HSD-1 could be blocked by the compound equilin by binding to its catalytic subunit (Sawicki et al., 1999). Since the presence of equilin did not altered the hormonal action either on NO production or VSMC proliferation, the fast and long term effect of E_1 would not be achieved to an estradiol action, consequent to an E_2 synthesis from E_1 sources. Moreover, we provided evidence that estrone treatment of RAS did not increase estradiol concentration in the incubation medium.

It is widespread considered that steroids hormones act via it classical mechanism of action regulating target genes transcription after binding nuclear receptors (Truss and Beato, 1993). However, the existence of non-genomic action of steroids has been widely accepted during the last decade (Wehling and Lösel, 2006). Estradiol as other steroid hormones is traditionally considered to transactivate target genes after binding nuclear receptors. However, E_2 also has rapid, non-genomic effects, and these have been attributed to cell membrane initiated signaling. At cell surface, a small population of ER binds E_2 and activates G proteins. Multiple signalling pathways are then rapidly stimulated by E_2 in target cells that express endogenous ERα and ERβ, and these pathways have been linked to specific actions of the steroid. In vascular endothelial cells, gene transcription and cell biological effects of estrogens emanate from rapid and specific signalling, integrating cell surface and nuclear action (Pedram et al., 2002). Phosphorylation of ERα by kinase cascades could provide a novel coupling mechanism between events at the membrane and transcriptional events in the nucleus (Vasudevan et al., 2005). Moreover, MAPK and PKC rapid stimulation could result in an enhanced phosphorylation of coactivators or transcriptional factors required for a fully steroid regulating of gene activation (Rowan et al., 2000).

In contrast with the large amount of data reported of vascular effect of E_2, the mechanism of action of E_1 on vascular tissue has been poorly investigated. Although various epidemiological studies have demonstrated a high incidence of cardiovascular events after menopause, it is also true that not all postmenopausal women develop atherosclerosis despite decreased levels of serum E_2 (Cauley et al., 1992). In view of the discrepancies about the atheroprotective effect of HRT that emerge from clinical trials, a current theory proposed that it may be caused by different metabolism of the administrated estrogen due to genetic differences. This is supported by the finding that E_2 metabolites at least in part mediate the cellular effect of E_2 (Barchiesi et al., 2002). Therefore, it is important to examine the role of other estrogens or estrogens metabolites. It has been proposed that E_1-sulfate and estriol (E_3) would exert an atheroprotective effect through the inhibition of the expression of growth factor and autocrine mediators involved in atherosclerosis (Kikuchi et al., 2000). In HUVEC cells, the estrogen metabolite 17-epiestriol, suppressed TNF-α induced VCAM-1 expression and these actions were modulated at least in part through NO (Mukherjee et al., 2003).

We demonstrated that E_1 acutely increased NO production and that this event was dependent on the activation of MAPK

signal transduction system, since the compound PD98059 completely blocked the hormonal enhancement of NO. Moreover, the results obtained with the phosphatase inhibitor calyculin A emphasized the relationship between phosphorylation and E_1-NO synthesis. The time course of E_1-NO production exhibited a biphasic profile, that would be due to a selective modulation of estrone NO production elicited by diverse transduction signal pathway, at the earlier or later nitric oxide rise. We have previously shown that estrone rapid action on vascular tissue involved a cross talk between NOS and COX system (Massheimer et al., 2006).

It is still uncertain whether E_1 posses its own receptor or acts via estradiol receptor. The ligand binding ability of E_1 to ERs had been studied by others authors. Estrone exhibited a 60% relative binding affinity respect to 17β estradiol when bind to rat ERα (Kuiper et al., 1997; Gruber et al., 2002). In calf uterine estrogen receptor, estrone occupied the same site as estradiol, with an increased receptor's cooperative binding dependent on ER concentration (Sasson and Notides, 1983). Using fluorescence polarization technique or a resonance biosensor, E_1 binding to human recombinant estrogen receptor have been also reported (Ohno et al., 2002; Usami et al., 2002). Nevertheless, the molecular characteristics of E_1–ER binding are not fully understood. In this work is that either the rapid effect of the steroid on NO synthesis or the genomic stimulation of VSMC proliferation was blunted by the presence of the specific ER antagonist ICI 182780, suggesting that the biochemical action of E_1 on vascular tissue would be mediated by ER. Study on E_1–ER interaction in our experimental systems, will be further investigated in future studies.

We showed that E_1 regulated VSMC growth by stimulating cell proliferation and migration, via a genomic mechanism of action. The proliferative effect was dependent on the hormonal activation of PLC/PKC/system. We have previously reported that E_1 rapidly enhanced PLC activity, eliciting a biphasic increase in diacylglicerol generation, but this event was not involved in E_1-NO production (Massheimer et al., 2006). The present results provide evidence that the mitogenic response of E_1 was dependent on the activation of PKC pathway, since the PKC inhibitor chelerythrine completely blocked the hormonal stimulus on ^{3}H-thymidine incorporation. Moreover, although estrone rapidly activated MAPK and PLC/DAG/PKC cascades, we do not found evidence that VSMC proliferation would involve a cross talk between both signalling pathways.

It has been demonstrated that 17-beta estradiol inhibited PDGF-dependent VSMC proliferation (Kappert et al., 2006). In this work, we showed that E_1 promoted VSMC proliferation and migration. At the present we have no explanation for this apparent discrepancy between estradiol and estrone effects. Perhaps both estrogens regulate in different manner the signalling pathways that conduct to VSMC proliferation, or may act at distinct subpopulations of smooth muscle cells that exist in the arteries (Hao et al., 2003). Since the data obtained either with E_2 or E_1 belongs from each individual action on VSMC, it would be also relevant to evaluate the hormonal effect when both estrogens will be added simultaneously.

VSMC proliferation and migration within the vascular wall play a crucial role in the formation and subsequent progression of atherosclerotic lesions. Our previous results (Sellés et al., 2005; Massheimer et al., 2006) have shown that E_1 stimulates the synthesis of NO and prostacyclin, two main factors required to maintain the vascular tone and to avoid vascular endothelial injury. However, at the present we are unable to determine whether the observed effect of the steroid on VSMC proliferation, represents a risk or benefit against vascular diseases. This fact will be the focus of our future investigations.

Acknowledgements

This research was supported by grants from the SGCyT, Universidad Nacional del Sur, Argentina (PGI 24/B093); Agencia Nacional de Promoción Científica (PICTO 527, ANPCYT/UNS); Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 5790 CONICET, Argentina) and Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC, Argentina). We thank Biochemistry Graciela Gigola for her collaboration with the inmunocytochemistry techniques. We dedicate this contribution to the memory of our recently deceased research teammate and friend, Dra. Juana Sellés.

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