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Cross-talk between rapid and long term effects of progesterone on vascular tissue

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ABSTRACT

We tested the hypothesis whether; the non-genomic action of progesterone (Pg) on vascular tissue would be associated with hormonal long term effect on the modulation of cell growth. Using rat aortic strips, we showed that the stimulatory effect of Pg on nitric oxide synthesis involved both kinase and phosphatase pathways. The increase in the vasoactive production was prevented by the MAPK inhibitor (PD98059). In addition, preincubation with a phosphatase antagonist potentiated the hormonal effect. Pg increased PKC activity, but the inhibition of PKC did not alter the stimulatory action of the hormone on nitric oxide generation. In endothelial cell cultures (EC), 24 h treatment with Pg significantly diminished cell proliferation. This antiproliferative effect was suppressed by the PKC inhibitor chelerythrine (chel) and L-NAME (nitric oxide synthase inhibitor). We also observed that Pg stimulates EC migration. In summary, the present findings provide evidence of an integration of genomic and non-genomic effects in the mechanism of action displayed by Pg in vascular tissue. The fast effects elicited by the hormone implies signal transduction activation required for the regulation of vasoactive production, but also necessary for the modulation of endothelial cells growth.

1. Introduction

Progesterone (Pg) and other steroid hormones are traditionally considered to transactivate target genes after binding nuclear receptors [1]. However, Pg also has rapid, non-genomic effects attributed to cell membrane-initiated signaling [2,3]. Non-genomic actions of Pg have been identified in several cell types such as hypothalamus, human sperm, hepatocytes, oocytes and in human amniochorion [4,5]. In the last few years, a two-step model for steroid action has been postulated. This model proposes that rapid actions at membrane level trigger intracellular transduction pathways activation that would converge in the synthesis of transcriptional factors required for the genomic action. Recent evidence shows that in rod bipolar cells, Pg could influence retinal function through the classical genomic mechanism and/or through the activation of signal pathways that may also converge on a nuclear end point [6].

Progesterone has been extensively used in hormone replacement therapy (HRT). Based on observational and mechanistic studies, it has been assumed that HRT possess cardio protective properties [7]. However, recent trials failed to show advantages in prevention of primary or recurrent cardiovascular events in women receiving HRT [8]. The reason for this is still unclear. Historically, progestins were included in HRT protocols to counteract endometrial dysplasia caused by estradiol. Lesser is known about the biochemical action of Pg on vascular physiology, in contrast to the large amount of evidence showing the direct effects of estrogens on cardiovascular system [7]. The presence of Pg receptor in the vascular tissue has been reported [9]. Evidence of vasorelaxation induced by Pg was obtained in rabbit coronary arteries [10] and in rat aorta, attributed to the inhibition of calcium entry or blockage of voltage-dependent and/or receptor-operated calcium channels [11]. Moreover, it has been reported that in human endothelial cells, Pg inhibits vascular cell adhesion molecule-1 (VCAM-1) synthesis induced by tumor necrosis factor-α (TNF-α) [12], and intercellular adhesion molecule (ICAM) expression in cells exposed to lipopolysaccharide [13].

The use of HRT has been proposed to prevent several postmenopausal disorders such as cardiovascular diseases. In western world, cardiovascular diseases mainly due to atherosclerosis continue to be the principal cause of death [14]. For vascular disorders prevention, the maintenance of the endothelial physiological properties is essential. Mechanical or chemical injury results in endothelial dysfunction and initiates atherosclerosis [15]. The early phase of atherosclerotic plaque generation is characterized by impaired synthesis of vasodilator molecules, such as nitric oxide (NO) and prostacyclin (PGI_2) [16], subsequently followed by morphological changes, enhanced cell adhesion

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molecules expression, and alterations in cell growth and migration [17,18].

Nitric oxide, prostaglandins, and thromboxane regulate vascular tone and vessel physiology [19]. NO is synthesized by the enzyme NO synthase (NOS) via the conversion of L-arginine to L-citrulline [20]. Endothelial cells constitutively express eNOS, isoform that requires calcium and calmodulin as cofactors [21]. NO is a potent vasodilator, which prevents platelet aggregation and neutrophil adhesion to the endothelium [22,23].

In our laboratory, we study the biochemical action of Pg on rat aortic tissue. We have previously demonstrated that Pg exerts a direct, non-genomic action on rat aortic metabolism, which includes nitric oxide synthase (NOS) and cyclooxygenase (COX) activation. The stimulation of vasoactive production activity was specific for the sexual female steroids Pg, estrone and estradiol. Furthermore, this non-genomic action of Pg was selective for female rats with intact ovarian function, since neither male nor ovariectomized rats exhibit any response to hormonal treatment [24,25]. The mechanism of action of Pg involves tyrosine kinase and phosphatidylinositol-3-kinase (PI3K) cascades and a cross-talk between NOS and COX systems [26]. The hormonal action not only implies the modulation of the synthesis of vasoactive compounds, but also involves vascular regulation of platelet aggregation, event dependent on PLC system activation [26]. Following with the study of Pg vascular action, the aim of the present work was to test the hypothesis whether Pg would act via a two-step mechanism of action that would involve interactions between genomic and non-genomic effects. To that end we evaluate the probability of an association between the Pg-non-genomic activation of signal transduction pathways, and its long term effect on cell growth regulation.

2. Materials and methods

2.1. Materials

Progesterone was obtained from Calbiochem-Novabiochem International (San Diego, CA), chelerythrine and calyculin A were gently donated by Alomone Labs. (Jerusalem, Israel). ^{3}H-Thymidine was purchased from New England Nuclear (Chicago). Griess reaction solutions were purchased from Britania Laboratories (Buenos Aires, Argentina). Trypsin/EDTA (10X), L-glutamine (100X), amphotericin B (0.25 mg/ml), penicillin/streptomycin (100X) and fetal bovine serum were obtained from PAA Laboratories (Pasching, Austria). PD98059, Dulbecco's modified Eagle's medium modified and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Sexually mature female Wistar rats (4–7 months old) that had progressed through at least three consecutive oestrous cycles were fed with standard rat food, given water *ad libitum* and maintained on a 12 h light/12 h dark cycle. The oestrous cycle activity 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. 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. Rat aortic strips (RAS) preparation

RAS were obtained as previously described [24]. Briefly, animals were killed by cervical dislocation and the thoracic aorta was immediately removed and placed in cold Dulbecco's Physiological Buffered Solution (DPBS), cleaned of adherent connective tissue, and cut in 15 mm strips. Special care was taken to avoid contact with luminal surface in order to preserve the endothelium integrity. The strips were placed in the incubation medium: 145 mM NaCl; 5 mM KCl; 1.2 mM $MgSO_4$; 1 mM $CaCl_2$; 10 mM glucose; 10 mM Hepes pH 7.35, and preequilibrated for 10 min at 37 °C in a shaking thermostatized water bath [26]. In vitro treatments were performed by incubation with the hormone for short time intervals. Control groups received vehicle alone (<0.1%). When inhibitors (PD98059, chelerythrine and calyculin A compounds) were employed, they were added to the incubation medium 30 min before treatment.

2.4. Rat aortic homogenates (RAH) preparation

RAS were obtained as previously indicated and they were mechanically homogenized in an appropriate buffer in order to perform the PKC and MAPK enzymatic assays; for PKC assay: 20 mM Tris–HCl pH 7.4, 0.33 M sucrose, 1 mM EGTA, 20 mM sodium fluoride (NaF), 1 mM dithiothreitol (DTT) and 20 μg/ml aprotinin; for MAPK assay: 20 mM Tris–HCl pH 7.4, 1 mM EGTA, 0.27 M sucrose, 50 mM NaF, 1 mM EDTA, 1 mM DTT, 2 μg/ml pepstatin, 2 μg/ml aprotinin and 1 mM sodium orthovanadate. After immediately, the homogenates were frozen at −80 °C until they were employed [27]. Alternatively RAH were obtained from RAS formerly exposed to Pg. To that end RAS were incubated with Pg for the time required for each experiment. The treatment was stopped by placing the tissue in liquid nitrogen. RAH were obtained by mechanically homogenized as described above, and frozen at −80 °C until they were employed in phosphorylation assays.

2.5. Endothelial cell cultures

EC cultures were obtained from aortic rings explants isolated from young Wistar female rats (3–5 weeks old) [28]. Briefly, the full length thoracic aorta was aseptically removed and then cut into ring segments (1.5 mm). Ring explants were seeded in 60-mm matrix-coated Petri-dishes (NUNC) containing phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 3.7 mg sodium bicarbonate, 100 U/ml penicillin, 10 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. The identity of the EC 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 bioability to synthesize nitric oxide [29]. Cells from passages 2–7 were used for all experiments.

2.6. Measurement of NO production

RAS were treated as indicated previously and 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 [30]. Briefly, aliquots of incubation medium were mixed with Griess reagent (1% sulphanilamide and 0.1% naphthylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated 10 min at room temperature. When EC were used, cells were seeded on 24 well plates (NUNC) at a density of 3.5×10^4 cells/well and allowed to grow to 90% confluence. After respective hormonal treatment in fresh DMEM containing 1% FBS, 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. The concentra-

tion of nitrite in the samples was determined with reference to a sodium nitrite standard curve performed in the same matrix. The aortic strips and cells were then dissolved in 1N NaOH and aliquots were taken for protein determination by Lowry method [31]. Results were expressed as nmol of NO per mg protein.

2.7. PKC activity assay

PKC activity was determined in RAH using a specific substrate phosphorylation assay as described previously [32]. In brief, reaction mixture containing: 100 μg protein (RAH), 20 mM Tris–HCl pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA, 20 mM NaF, 1 mM DTT, 20 μg/ml aprotinin, 5 μg histone (type III-S) with or without 1 mM $CaCl_2$, 10 μg phosphatidylserine (PS) and 0.75 μg di-acyl glycerol (DAG) was incubated with different concentrations of hormone or vehicle control (<0.1%) for short time intervals. The addition of the hormone was avoided when RAH were isolated from RAS formerly treated with Pg. ATP–ATP-[γ^{32}P] (100 μM; 0.2 μCi) was added for 5 additional minutes. The reaction was stopped by transferring an aliquot of assay mixture into phosphocellulose disk (Whatmann P-81) and then they were washed four times by shaking in 30% acetic acid/1% phosphoric acid mixture, rinsed once with 96% ethanol and air dried. The radioactivity was quantified by liquid scintillation using a Wallac1414 counter (PerkinElmer Winspectral, Wellesley, USA) and the enzymatic activity was calculated beginning from the difference between phosphorylation assessed in the presence and the absence of Ca, PS and DAG. Results were expressed in pmol of P/min per mg protein.

2.8. MAPK activity assay

Rat aortic homogenates were immunoprecipitated with anti-MAPK kinase (p42 and p44 fractions), extensively washed, and an aliquot was suspended in 100 μl of a reaction mixture containing: 25 mM Tris–HCl pH 7.0, 5 mM $MgCl_2$, 100 μM EGTA, 100 μM sodium orthovanadate, 20 μg/ml pepstatin, 20 μg/ml aprotinin, 1 mM DTT, 1 μM okadaic acid and 400 μg/ml myelin basic protein (MBP) as an exogenous substrate for MAPK. Treatment was performed by incubation with different concentrations of hormone or vehicle control (<0.1%) for short time intervals. The addition of the hormone was avoided when RAH were isolated from RAS formerly treated with Pg. ATP–ATP-[γ^{32}P] (100 μM; 0.2 μCi) was added to assay mixture and incubated for 15 additional minutes. The reaction was stopped by separating the phosphorylated substrate on phosphocellulose disks (Whatman P-81) which were then washed four times by shaking in 0.5% phosphoric acid, rinsed once with 96% ethanol and air dried. The radioactivity was quantified by liquid scintillation using a Wallac1414. Results were expressed in pmol of P/min per mg protein [27].

2.9. [^{3}H]-Thymidine incorporation assay

Endothelial cells were seeded on 24 multi-well plates (NUNC) at a density of 3×10^4 cells/well in DMEM supplemented with 10% FBS and allowed to grow to 60–70% confluence. The cells were made quiescent by placing in serum-free DMEM for 24 h and further exposed to different concentrations of Pg or vehicle control (<0.1%) for 24 h in fresh DMEM containing 1% FBS. When the compounds chelerythrine, L-NAME, actinomycin D and cycloheximide were used, they were added 1 h before hormonal treatment. The cells were pulsed with 1 μCi/ml of [^{3}H]-thymidine during the last 2 h of 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 dissolved with 1N NaOH. Radioactivity was measured by liquid scintillation using a Wallac1414 counter. The protein concentrations were determined by Lowry method and the results were expressed as cpm/per mg protein [33].

2.10. Endothelial cells migration assay

Briefly, endothelial 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 24 h in 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 progesterone or vehicle control (<0.1%). After 48 h of culture, the cells were fixed in glutaraldehyde 0.1% and stained with haematoxylin–eosin. Migration was quantified by counting the number of cell nuclei that crossed the line demarcated in at least seven different microscopic fields (×40 and ×100) representative of each culture plate. Results are expressed as means ± SD of number of cells/field [34,35].

2.11. Statistical analysis

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

We have previously reported that tyrosine kinase and PLC transduction system are involved in the non-genomic action of Pg in vascular tissue [25]. Following this line of investigation we studied the direct effect of Pg on MAPK and PKC activity. To that end, rat aortic homogenates were exposed to different concentration of Pg, and PKC activity was measured using a specific substrate phosphorylation assay. Fig. 1 shows that the steroid elicited a dose dependent

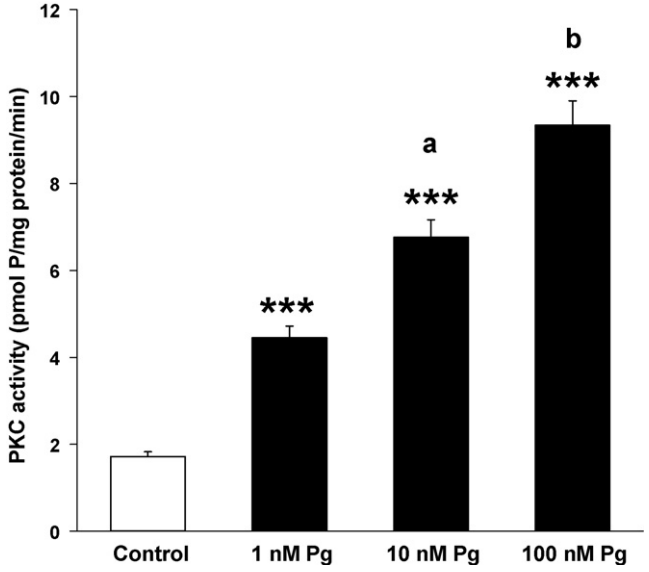

Fig. 1. Stimulation of PKC activity induced by Pg: dose response profile. Rat aortic homogenates were treated with Pg at the concentrations indicated for 10 min. After immediately, 10 μl of 100 μM ATP/[γ^{32}P]–ATP (0.2 μCi) were added for 5 additional minutes. PKC activity was measured as described under Section 2. Results are the average ± SD of three independent experiments performed by quadruplicate. $^{***}p < 0.001$ respect to control value; $^{a}p < 0.02$ respect to 1 nM Pg; $^{b}p < 0.001$ respect to 10 nM Pg.

Table 1
Effect of progesterone on PKC and MAPK activities.

Treatment	PKC activity (pmol P/mg protein/min)	MAPK activity (pmol P/mg protein/min)
Control	2.51 ± 0.26	4.76 ± 0.57
10 nM Pg	7.32 ± 0.80[b]	8.33 ± 0.41[b]

Rat aortic strips were incubated with 10 nM Pg for 10 min. RAH were obtained by mechanically homogenized, and phosphorylation assays were performed as described under Section 2. Results are the average ± SD of three independent experiments ($n = 4$).

[b] $p < 0.02$ respect to each control value

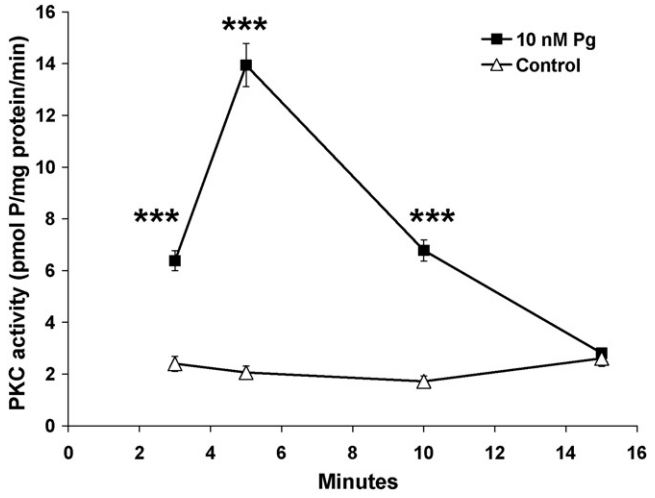

Fig. 2. Stimulation of PKC activity induced by Pg: time response profile. Aortic homogenates were exposed to 10 nM Pg at the indicated times. Immediately after, 10 μl of 100 μM ATP/[γ^{32}P]–ATP (0.2 μCi) was added for 5 additional minutes. PKC activity was measured as described under Section 2. Results are the average ± SD of three independent experiments ($n = 4$). $^{***}p < 0.001$ respect to each control value.

stimulatory action on PKC activity. As can be observed, ten minutes treatment with Pg (1–100 nM) significantly induced 2-fold to 4-fold incremental increase in PKC activity. Statically differences were detected among each concentrations tested. Table 1 shows the results obtained when the phosphorylation assays were performed using RAH isolated from RAS formerly exposed to Pg. As can be observed the hormonal treatment induced significant stimulation of PKC and MAPK activities. Since this results were similar to those obtained with direct steroid treatment of RAH, in order to avoid twice frozen, the following experiments were done by direct adding of Pg to RAH. The time response profile shows that the stimulatory action of the hormone was already seen at 3 min treatment and sustained after 10 min of exposure to the hormone, diminishing to control values at 15 min (Fig. 2). In MAPK assays, we found that Pg (1–100 nM) significantly increased MAPK activity (35–66% above control, Fig. 3). The enhancement on this kinase activity was detected between 1 and 10 min of hormonal treatment (Table 2).

Table 2
Effect of progesterone on MAPK activity.

Treatment	MAPK activity (pmol P/mg protein/min)		
	1 min	10 min	15 min
Control	5.89 ± 0.49	5.00 ± 0.88	4.46 ± 1.43
10 nM Pg	8.57 ± 0.85[b]	9.69 ± 0.90[b]	4.01 ± 0.92

Rat aortic homogenates were treated with 10 nM Pg at the indicated times. Immediately after10 μl of 100 μM ATP/[γ32P]–ATP (0.2 μCi) were added for 15 additional minutes. MAPK activity was measured as described under Section 2. Results are the average ± SD of three independent experiments ($n = 4$).

[b] $p < 0.02$ respect to each control value.

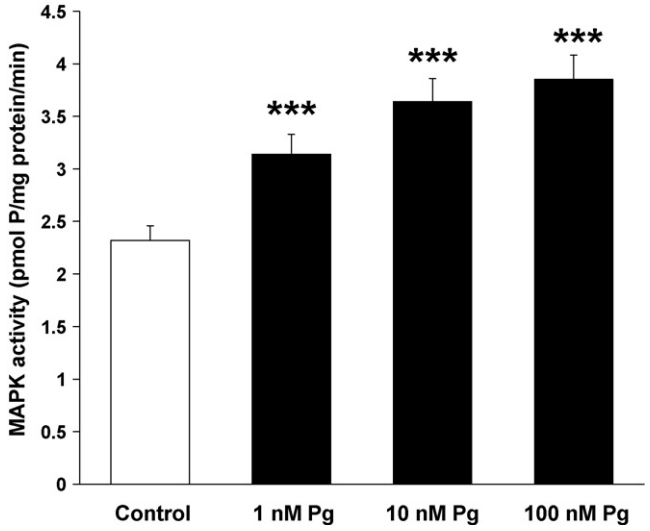

Fig. 3. Dose response profile of stimulation of MAPK activity induced by Pg. RAH were treated with Pg at the concentrations indicated for 10 min. After immediately, 10 μl of 100 μM ATP/[γ^{32}P]–ATP (0.2 μCi) were added for 15 min. MAPK activity was measured as described under Section 2. Results are the average ± SD of three independent experiments ($n = 4$). $^{***}p < 0.001$ respect to control value.

Afterwards, in order to check the role of MAPK and PKC transduction systems in the stimulation of nitric oxide production induced by Pg, specific inhibitors were employed (compounds PD98059 and chelerythrine for MAPK and PKC respectively). Rat aortic strips were preincubated with 5 μM PD98059, and immediately after exposed to 10 nM Pg for 5 min treatment. Table 3 shows that the presence of the blocker completely suppressed the increase in NO synthesis elicited by the hormone. However, the blockage of PKC activity with 1 μM chelerythrine did not alter the stimulatory action of Pg (Table 3).

Taking in account that, cellular signalling involves either phosphorylation or desphosphorylation mechanism, we evaluated the effect of phosphatases on the stimulation of NO production induced by Pg. We chose the compound calyculin A (Cal) as a potent inhibitor of phosphatase PP1 and PP2A. In the presence of the inhibitor (Table 3) the stimulus on nitric oxide synthesis was significantly enhanced (193 vs. 53% above control, Pg with or without Cal, respectively). This data reinforce the hypothesis that the stimulatory effect of Pg on NOS activity would be mediated both by kinase and phosphatase pathways.

The experiments described above were performed using rat aortic strips as experimental model. RAS contain both endothelial and smooth muscle cells. In order to study the Pg action at endothelial level we performed endothelial cell cultures. Firstly, we measured nitric oxide production under hormonal treatment in these isolated

Table 3
Role of kinases and phosphatases pathways on the stimulation of nitric oxide production induced by Pg.

Treatment	NO production (nmol/mg protein)			
		+PD98059	+Chelerythrine	+Calyculin A
Control	0.471 ± 0.100	0.486 ± 0.070	0.452 ± 0.040	0.470 ± 0.027
10 nM Pg	0.843 ± 0.135[b]	0.503 ± 0.025	0.924 ± 0.120[b]	1.378 ± 0.020[a,c]

RAS were pre-incubated for 30 min in presence or absence of 5 μM PD98059, 1 μM chelerythrine or 0.1 nM calyculin A and then exposed to 10 nM Pg for 5 min. NO production was measured by Griess reaction as described under Section 2. Results are the average ± SD of three independent experiments performed by quadruplicate.

[a] $p < 0.05$ vs. Pg alone.

[b] $p < 0.02$.

[c] $p < 0.001$ respect to each control value.

Table 4
Effect of progesterone on NO production in EC.

Treatment	NO production (nmol NO/mg protein)			
	Control	1 nM Pg	10 nM Pg	100 nM Pg
	20.56 ± 0.65	29.19 ± 0.67[b]	26.52 ± 5.25[b]	41.73 ± 5.78[b]

EC cultured in presence of FBS 1% were incubated with Pg at the indicated concentrations for 20 min. NO production was measured by Griess reaction as described under Section 2. Results represent the average ± SD of three independent experiments ($n = 4$).

[b] $p < 0.02$ respect to control value

cells. We found that, similarly as in RAS, in EC, Pg induced a very fast stimulation of nitric oxide synthesis (Table 4).

Afterwards, in order to investigate the genomic effects of Pg, we tested the hormonal regulation of EC growth. Fig. 4 shows that after 24 h of Pg treatment, DNA synthesis was significantly inhibited (63–23% of inhibition) in a large range of steroid concentration (1–100 nM Pg). This antiproliferative effect of Pg was suppressed in the presence of the antagonist of PgR, compound RU486. As shown in Fig. 5, the blockage of the antiproliferative steroid action was observed in a wide range of antagonist concentration (10 nM to 10 μM). When EC were preincubated with gene transcription or protein synthesis inhibitors (actinomycin D; cycloheximide), Pg was unable to exhibit its antiproliferative action (Table 5). In the presence of actinomycin D or cycloheximide, the 53% of inhibition on ^{3}H-thymidine incorporation induced by Pg was not detected, suggesting the genomic feature of this effect.

Finally, we studied the existence of interactions between genomic and non-genomic effects of the sexual steroid. We obtained evidence that, the PKC cascade was involved in this genomic action of Pg on EC proliferation. Preincubation of EC with 1 μM chelerythrine, abolished the inhibition of ^{3}H-thymidine incorporation induced by 24 h of exposure to 10 nM Pg (Fig. 6). Moreover, the presence L-NAME, an NOS specific inhibitor, also blunted the inhibition of DNA synthesis induced by Pg (Fig. 6). Taken together, these results suggest that the genomic action of Pg on EC proliferation depends on PKC and NOS signalling pathways activation.

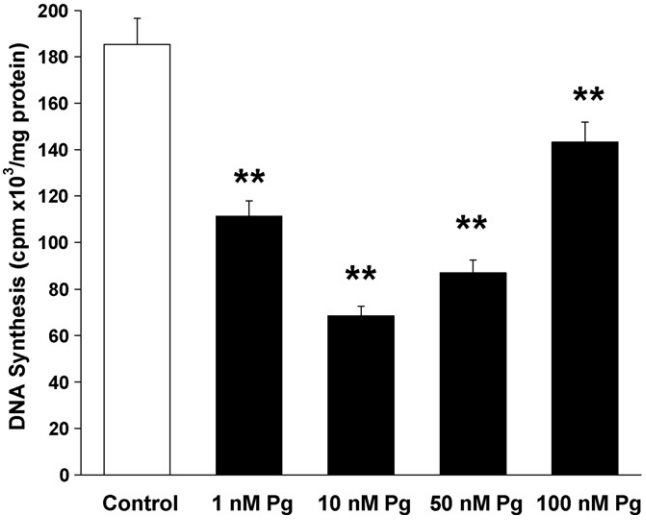

Fig. 4. Effect of progesterone on [^{3}H]-thymidine incorporation: dose response profile. Sub-confluent EC were incubated in serum-free medium for 24 h and then treated with Pg at the indicated concentrations for another 24 h. 1 μCi/ml of [^{3}H]-thymidine was added during the last 2 h of treatment. [^{3}H]-Thymidine incorporation was measured as described in Section 2. Results represent the average ± SD of three independent experiments performed by quadruplicate. $^{**}p < 0.02$ respect to control value.

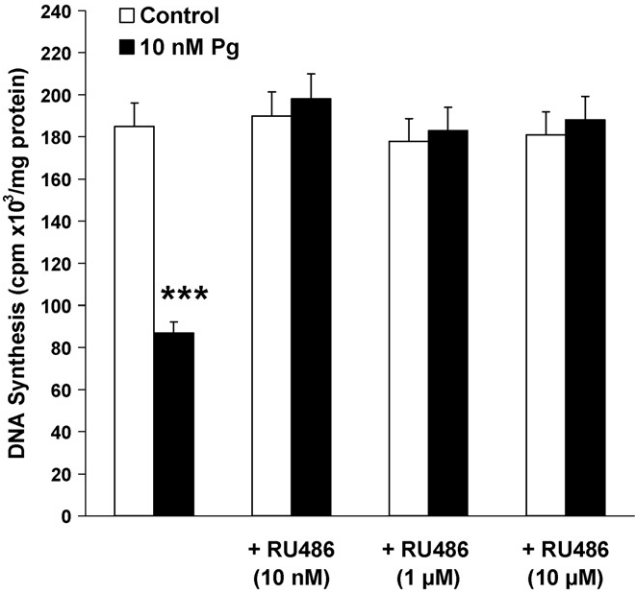

Fig. 5. Effect of RU486 on Pg-regulated EC [^{3}H]-thymidine incorporation. Sub-confluent EC were incubated for 24 h in serum-free media, pre-incubated in absence or presence of the indicated concentrations of RU486, and then treated with 10 nM Pg for 24 h. 1 μCi/ml of [^{3}H]-thymidine was added during the last 2 h of treatment. [^{3}H]-Thymidine incorporation was measured as described in Section 2. Results represent the average ± SD of three independent experiments ($n = 4$). $^{***}p < 0.001$ respect to control value.

Table 5
Effect of cycloheximide and actinomycin D on ^{3}H-thymidine incorporation elicited by Pg in EC.

Treatment (24 h)	DNA synthesis (cpm × 10^3/mg protein)			
		+10 μM Cycloheximide	+50 μM Cycloheximide	+1 μg/ml actinomycin D
Control	196 ± 39.2	167 ± 33.4	157 ± 30.4	153 ± 20.4
10 nM Pg	91 ± 22.31[b]	161 ± 22.3	160 ± 20.8	155 ± 17.1

EC cultured in presence of FBS 1% were incubated with 10 nM Pg for 24 h. Cycloheximide (10; 50 μM) and actinomycin D (1 μg/ml) were added to the incubation medium 1 h prior hormonal treatment. ^{3}H-Thymidine incorporation was measured as described under Section 2. Results are the average ± SD of three independent experiments ($n = 4$).

[b] $p < 0.02$ respect to each control value.

Since in several vascular disorders, cell migration is an important physiological event involved in tissue healing, we study the effect of Pg on endothelial cells migration. Fig. 7 shows a microphotography of a representative assay. It can be observed the cells that crossed the line demarcated and migrated to the denuded area after 48 h treatment with 10 nM Pg or vehicle alone (control). The data provides evidence that Pg stimulates cell migration, inducing a 3.5-fold enhancement in EC movement.

4. Discussion

The results presented in this work show that, on vascular tissue, progesterone exerts both rapid and long term effects; the former related to steroid activation of PKC, MAPK and NOS; and the latter associated with inhibition of endothelial cell proliferation. The stimulation of nitric oxide production was dependent on MAPK cascade participation, but independent of PKC pathway. The genomic steroid action displayed by Pg includes the inhibition of EC proliferation, and this antiproliferative effect involves the participation of PgR, and the activation of PKC and NOS transduction system.

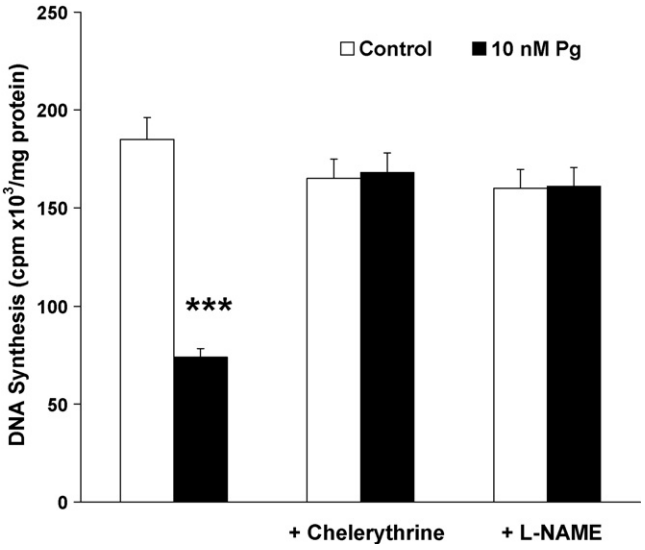

Fig. 6. Effect of chelerythrine and L-NAME on Pg-regulated [^{3}H]-thymidine incorporation in EC. Sub-confluent EC were incubated for 24 h in serum-free media. Chelerythrine (1 μM) or L-NAME (10 μM) were added to the incubation medium 1 h prior treatment with 10 nM Pg for additional 24 h. 1 μCi/ml of [^{3}H]-thymidine was added during the last 2 h of treatment. [^{3}H]-Thymidine incorporation was measured as described in Section 2. Results represent the average ± SD of three independent experiments ($n = 4$). $^{***}p < 0.001$ respect to control value.

It is widespread considered that steroids hormones act via it classical mechanism of action regulating target genes transcription after binding nuclear receptors [36]. However, the existence of non-genomic actions of steroids have been widely accepted during the last decade [37]. Integrating genomic and non-genomic actions, the two-step model of steroid action propose the modulation of classical receptor induce gene transcription by non-genomic signal transduction pathways. This model has been originally developed for aldosterone [38] and later expanded to others steroids [4]. It has been reported that MAPK and PKA rapid stimulation could result in an enhanced phosphorylation of co-activators or transcriptional factors required for chicken Pg receptor modulation [39]. In vascular endothelial cells, gene transcription and cell biological effects of estrogens emanate from rapid and specific signalling, integrating cell surface and nuclear action [35]. At the present, we don't have determined the target genes of Pg action in our experimental system. This will be the focus of our future investigations. Nevertheless, the data reported in this work, provides evidence of one more steroid action that would be displayed via the two-step model.

On the non-genomic study we fixed our attention on MAPK and PKC transduction systems. We have previously demonstrated that Pg exerts a direct non-genomic action on rat aortic metabolism, which involves NOS activation and regulation of platelet aggregation [25], mediated in part through tyrosine kinase and PLC system respectively. The classical PgR did not participate in this non-genomic action [26]. The present study demonstrated that Pg stimulates MAPK activity, and that this transduction system contributes with the hormonal stimulation of NO synthesis. Linkage between MAPK cascade and eNOS activation has been also established in different experimental models [40,41]. Indeed, in HUVEC the presence of the MEK1/2 inhibitor, blocked NOS stimulation elicited by 30 min treatment with Pg [13]. On the other hand, we showed that Pg increases PKC activity, with a temporal profile similar to the time course of Pg-DAG production previously reported [26]. In agreement with this observation, PKC activation by Pg has been reported in other experimental systems such as human sperm and bovine luteal cells [2,42]. We don't found association between Pg-PKC activation and nitric oxide production.

In order to investigate the genomic action of Pg we selected the regulation of EC growth. Endothelial cells play a pivotal role

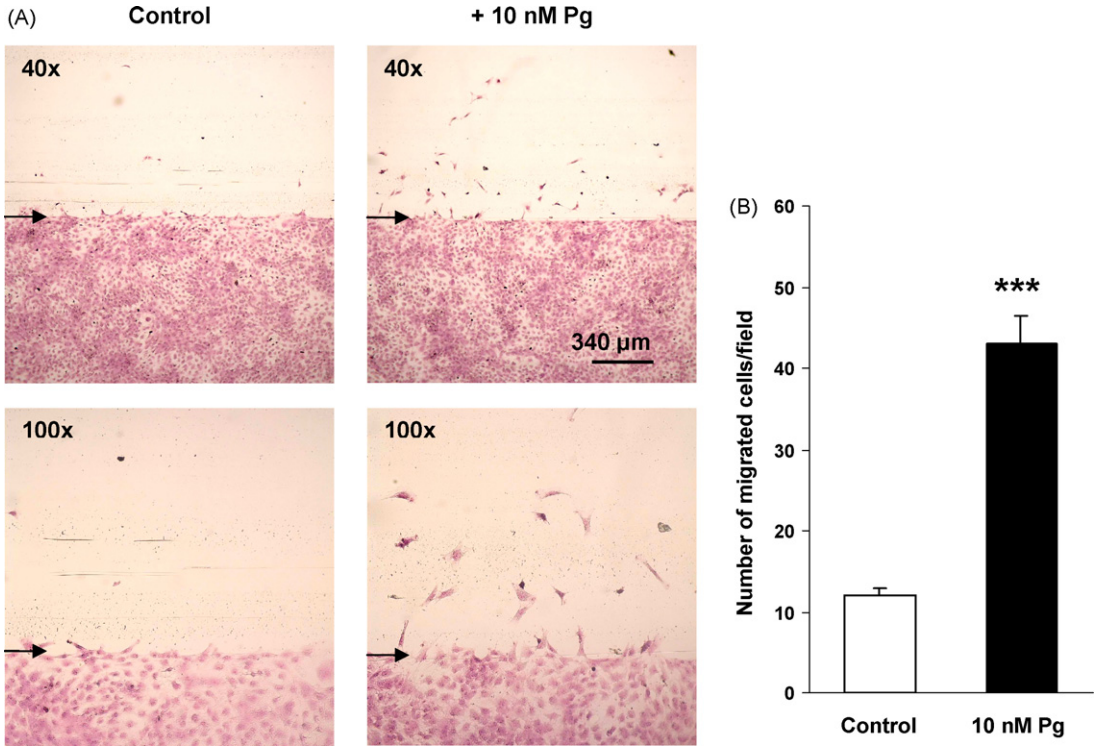

Fig. 7. Effect of Pg on endothelial cell migration. Confluent EC cultured on 60-mm dishes were serum starved for 24 h. After scraping the cells besides the wound (indicated by arrows), the half remaining monolayer were treated with 10 nM Pg for another 48 h and then processed as described in Section 2. (A) Images captured after haematoxylin–eosin staining (40× and 100× magnification of same field). (B) Bars represent the means ± SD of number of migrated cells/field from three separated experiments performed by quadruplicate. $^{***}p < 0.001$ compared with control.

in vascular physiology since they regulate a plethora of vascular functions. It is therefore conceivable that the maintenance of the endothelial layer physical continuity and function is crucial for the prevention of atherosclerosis and vascular diseases [43]. We showed that Pg inhibited rat aortic EC proliferation. The genomic nature of this effect was supported by the suppression of the antiproliferative effect of Pg with transcriptional and protein synthesis inhibitors. The presence of a PgR antagonist, the compound RU486, blunted the antiproliferative effect of Pg, suggesting that this genomic action of the steroid on EC growth modulation would be mediated by PgR. In agreement with this, in several independent strains of EC, receptor dependent inhibition of EC growth after 48 h of Pg treatment has been reported (9). An important finding of this work was the evidence that PKC and NOS cascades were involved with the long term effect of Pg on the inhibition of EC proliferation. In vascular smooth muscle cells (VSMC), nitric oxide prevents cell proliferation, through the induction of a G0/G1 cell cycle arrest [44]. Indeed, evidence of inhibition of cell growth mediated by PKC has been reported in HUVEC [45,46].

Perhaps, the antiproliferative effect of Pg may be considered as a deleterious fact for the endothelial healing and recovery after injury. Proinflammatory action of Pg has been reported in postmenopausal women through the potentiating of the IL-6-mediated stimulation of C-reactive protein, a marker of inflammation linked to the development of cardiovascular disorders [47]. However, when we examined the effect of Pg on EC migration, we found that Pg stimulates the EC movement into the denuded area, fact that could be associated with an improvement of remodelling or tissue repair action. Although we provide evidence that Pg modulates EC proliferation and migration through its direct action on EC cultures, the physiological relevance of this hormonal effect must be further investigated in future studies.

In summary, the present findings provide evidence of an integration of genomic and non-genomic effects in the mechanism of action displayed by Pg in vascular tissue. The fast effects elicited by the hormone implies signal transduction activation required for the regulation of vasoactive production, but also necessary for the modulation of endothelial cell growth. Although the existence of non-genomic effects for steroid hormones have been fully accepted in the last decade, the two-step model of steroid action arises as a new theory that integrates a cross-talk between classical and non classical actions. Our present work offers additional data that would support this postulation.

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