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Role of progesterone on the regulation of vascular muscle cells proliferation, migration and apoptosis

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ABSTRACT

The purpose of this study was to investigate the effect of progesterone (Pg) on cellular growth, migration, apoptosis, and the molecular mechanism of action displayed by the steroid. To that end, rat aortic vascular smooth muscle cell (VSMC) cultures were employed. Pg (10 nM) significantly increased [^{3}H]thymidine incorporation after 24 h of treatment. The enhancement in DNA synthesis was blunted in the presence of an antagonist of Pg receptor (RU486 compound). The mitogenic action of the steroid was suppressed by the presence of the compounds PD98059 (MEK inhibitor), chelerythrine (PKC inhibitor), and indomethacin (cyclooxygenase antagonist) suggesting that the stimulation of DNA synthesis involves MAPK, PKC, and cyclooxygenase transduction pathways. The proliferative effect of the hormone depends on the presence of endothelial cells (EC). When muscle cells were incubated with conditioned media obtained of EC treated with Pg, the mitogenic action of the steroid declined. Wounding assays shows that 10 nM Pg enhances VSMC migration and motility. The role of the steroid on programmed cell death was measured using DNA fragmentation technique. Four hours of treatment with 10 nM Pg enhanced DNA laddering in a similarly extent to the apoptotic effect induced by the apoptogen hydrogen peroxide (H_2O_2). In summary the results presented provide evidence that Pg enhances cell proliferation, migration, and apoptosis of VSMC. The modulation of cell growth elicited by the steroid involves integration between genomic and signal transduction pathways activation.

1. Introduction

The regulation of cellular behaviors, such as proliferation, differentiation, migration, and apoptosis plays an important role in many tissues. VSMC maintain vessel tone and blood pressure. Their functions and activities in the artery, both in normal and diseases states, are dependent on microenvironment created by the surrounding cells, soluble factors, and components of the extracellular matrix. VSMC exist in the normal blood vessel wall in a quiescent differentiated state with low rate of cell proliferation and turnover. Inflammatory or mechanical injuries to the artery wall, cause VSMC dedifferentiation, decrease of contractile properties, and induction of a proliferative and often motile phenotype [1,2]. Apoptosis is a form of cell death characterized by ordered dismantling of the cell through defined biochemical pathways and may occur in response to a wide range of stimuli [3]. This programmed cell death is part of normal development and senescence. Regulation of apoptosis is often altered in diseases states. Under vascular damage, the endothelium denudation would compromise the proper regulation of VSMC growth, proliferation, migration, and apoptosis [4]. Overall, regulation of VSMC phenotypes is important both at homeostasis and in the development of vascular diseases.

The incidence of cardiovascular and noncardiac vascular disease is greater in men compared with age matched premenopausal women. However during menopause this incidence increases dramatically. There is a long-standing hypothesis that estrogen levels might contribute to provide vascular protection. However the results of clinical trials raise an important controversy about the risk/benefit of hormone replacement therapy (HRT) [5]. Although the direct effects of estrogen on cardiovascular system are well recognized [6], much less is known regarding the vascular actions of progestins. The presence of progesterone receptor (PgR) either in

Abbreviations: 17β-E_2, 17β-estradiol; COX, cyclooxygenase; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; E2F1, E2F transcription factor 1; EC, endothelial cells; ERK1/2, extracellular signal-regulated kinase 1/2; FCS, fetal calf serum; HRT, hormone replacement therapy; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MPA, medroxyprogesterone acetate; NO, nitric oxide; NOS, nitric oxide synthase; PDGF, platelet-derived growth factor; Pg, progesterone; PGI_2, prostaglandin I2; PgR, progesterone receptor; PGRMC1, progesterone receptor membrane component 1; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; VSMC, vascular smooth muscle cells.

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endothelium and muscle cells has been reported [7]. Both isoforms of the classical nuclear PgR (PgR-A and PgR-B) have been identified [8]. Furthermore, the existence of the putative progestin receptor membrane protein component 1 (PGRMC1) has also been proposed [9].

Steroid hormones are traditionally known to exert their action through binding to steroids receptors, which are ligand-inducible transcriptional factors. However, an alternative mechanism of action, named non-genomic is also well recognized. These actions are characterized by rapid time-lapse of activation, insensitivity to transcriptional inhibitors and do not required nuclear localization of the receptor [10]. There is growing evidence that sex steroids regulate vessel function not only by long term genomic actions, but also triggering non-genomic action, such as vasodilatation via a rapid synthesis of nitric oxide [6]. Moreover, non-genomic actions are related to long term effects through a complex cross-talk between signal transduction pathways and steroid hormone activation [11].

In an attempt to contribute to the understanding of the role of progesterone (Pg) on vascular homeostasis, we developed a line of investigation that studies the biochemical action of Pg on rat aortic tissue. We found evidence 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 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 or ovariectomized rats exhibit any response to hormonal treatment [12,13]. This non-genomic action involves tyrosine kinase and phosphatidylinositol 3-kinase (PI3K) cascades, and a cross-talk between NOS and COX systems [14]. 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 phospholipase C (PLC) system activation [14]. We have recently reported an integration of genomic and non-genomic effects of Pg on vascular endothelium. The fast effects elicited by the hormone imply signal transduction activation required for the regulation of vasoactive production, but also necessary for the modulation of endothelial cells growth [15]. The purpose of our present study was to investigate the effect of Pg on the main cellular processes of VSMC function, such as growth, migration, and apoptosis. The molecular mechanism of the steroid action was also evaluated.

2. Experimental

2.1. Materials

Progesterone was obtained from Calbiochem-Novabiochem International (San Diego, CA), [^{3}H]thymidine was purchased from New England Nuclear (Chicago). Trypsin/EDTA (10×), L-glutamine (100×), amphotericin B (0.25 mg/ml), penicillin/streptomycin (100×), and fetal bovine serum were obtained from PAA Laboratories (Pasching, Austria). PD98059, Dulbecco's modified Eagle's medium modified [16]; chelerythrine, indomethacin, and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Female Wistar rats were fed with standard rat food, given water *ad libitum* and maintained on a 12 h light/12 h dark cycle. All animal work was performed at the Unit of Animal Care belonging to the Biology, Biochemistry and Pharmacy's Department of the National University of South. The Animal Care Use Committee approved the protocol used.

2.3. Endothelial and vascular smooth muscle cells cultures

EC and VSMC cultures were obtained from aortic rings explants isolated from young Wistar female rats (3–5 weeks old) [17]. Briefly, the full length thoracic aorta was aseptically removed and then cut into ring segments (2 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 20% (v/v) foetal calf serum (FCS), 3.7 mg sodium bicarbonate, 100 U/ml penicillin, 10 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L-glutamine. Explants were incubated at 37 °C in 5% CO_2 atmosphere. In order to establish a pure EC culture, after 3 days of culture, ring explants were removed and transferred into new culture dishes with fresh DMEM supplemented with 10% (v/v) FCS. Transfer of the same rings into new culture dishes at 5-day intervals then resulted in the progressive development of mixed cell populations (EC plus VSMC). Additional transfer of the ring explants resulted in a pure culture of VSMC. At last, the rings were discarded and EC and VSMC cultures were allowed to reach confluence. EC identification was performed by: (a) by phase-contrast microscope observation of the characteristic morphology of cobblestone shape growth in confluent monolayers, (b) by 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. The identity of the VSMC was determined by the positive inmunocytochemistry reactivity to smooth muscle specific alpha-actin, using DakoCytomation EnVision system [15,18]. Cells from passages 2–7 were used for all experiments. All hormone solutions employed in the in vitro cellular treatments were prepared using isopraponol as solvent. The final concentration of the vehicle was always below than 0.1%.

2.4. [^{3}H]Thymidine incorporation assay

Vascular smooth muscle cells were seeded on 24-multi-well plates (NUNC) at a density of 3×10^4 cells/well in DMEM supplemented with 10% (v/v) FCS 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 for 24 h in fresh DMEM containing 1% (v/v) FCS. When the compounds chelerythrine, PD98059, RU486, and indomethacin 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 phosphate-buffered saline (PBS) to remove the unincorporated [^{3}H]thymidine. Ice-cold trichloroacetic acid (10%, w/v) was added and the acid-insoluble material was dissolved with 1 M NaOH. Radioactivity was measured by liquid scintillation using a Wallac1414 counter. The protein concentrations were determined by the Lowry method [19] and the results were expressed as cpm per mg protein [20].

2.5. Proliferation assay using EC-conditioned media

EC cultures at 60–70% confluence were rinsed twice with PBS and incubated with 10 nM Pg or vehicle in DMEM containing 1% (v/v) FCS for 24 h at 37 °C. The EC media was collected by centrifugation at 500 × *g* for 10 min and frozen at −80 °C until usage. VSMC previously kept in serum-free medium for 24 h were then incubated with the conditioned media and [^{3}H]thymidine incorporation was measured as described above [21].

2.6. Cells migration assay

In brief, vascular smooth muscle cells (3×10^5) were seeded in 60-mm (NUNC) dishes with DMEM containing 10% (v/v) FCS and

grown to confluence. The cells were then 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% (v/v) FCS plus Pg or vehicle control. After 48 h of culture, the cells were fixed in glutaraldehyde 0.1% (v/v) 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×) representative of each culture plate. Results are expressed as means ± S.D. of number of cells/field [22,23].

2.7. Apoptosis assay

To assess the effect of Pg on cellular apoptosis, [^{3}H]thymidine apoptosis assays were employed [24,25]. Subconfluent cells (90%) seeded in 6-multi-well plates (NUNC) were labelled with [^{3}H]thymidine (1 μCi/ml) for 2 h before hormonal treatment. Cells were extensively washed with PBS to remove unincorporated [^{3}H]thymidine, and 10 nM Pg or vehicle alone were added for 4 additional hours. When 1 mM H_2O_2 was used, it was added 2 h before the end of hormonal treatment. Cells were lysed overnight at 37 °C in a specific buffer (10 mM EDTA/400 mM NaCl/10 mM Tris–HCl, pH 8.0/1% (v/v) SDS (sodium dodecyl sulfate)/1 mg/ml proteinase K), and immediately after, fragmented DNA was separated by centrifugation at 13,000 × *g* for 15 min at 4 °C. The presence of total or fragmented DNA in the precipitated or supernatant respectively, was checked by electrophoresis in agarose gel. Radioactivity of each fraction was quantified by triplicates. Results are expressed as % of fragmented DNA/total DNA.

2.8. Measurement of nitric oxide production

EC were seeded on 24-well plates (NUNC) at a density of 3.5×10^4 cells/well and allowed to grow to 90% confluence in DMEM containing 1% FCS. Hormonal treatment was performed by the addition of 10 nM Pg for 5 min. Nitrites (NO_2^-) were measured in the incubation media as a stable and non-volatile breakdown product of the nitric oxide (NO) released, employing Griess reaction [15]. Cells were dissolved in NaOH, and protein content was measured [19]. The results were expressed as nmol of NO_2^- per mg protein.

2.9. Statistical analysis

The results presented were obtained from at least three independent experiments where each individual experimental condition was performed by quadruplicate ($n = 4$) or quintuplicate ($n = 5$). Different cell cultures were used for each independent experiment. All data are presented as mean ± S.D. 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 [26].

3. Results

First we evaluated the effect of Pg on VSMC proliferation. To that end, synchronized cultures were exposed to 10 nM Pg in the presence of 1% (v/v) FCS. As can be observed in Table 1, 24 h of treatment with the steroid increased [^{3}H]thymidine incorporation (90% above control, $p < 0.02$). No significant differences between control and treated groups were observed after 48–96 h of treatment. The mitogenic action of the steroid was detected between 10 and 100 nM (90–30% above control), lower concentrations of the steroid were unable to modify DNA synthesis (Fig. 1A). In order to

Table 1
Time response profile of [^{3}H]thymidine incorporation elicited by Pg.

Treatment	DNA Synthesis (cpm × 10^3/mg protein)			
	24 h	48 h	72 h	96 h
Control	30.9 (±3.8)	12.9 (±1.7)	7.4 (±0.9)	8.1 (±0.5)
10 nM Pg	58.8 (±6.0)**	10.6 (±1.3)	7.8 (±2.8)	9.3 (±0.5)

Subconfluent VSMC were incubated in serum-free medium for 24 h and then treated with 10 nM Pg or vehicle at the indicated times. 1 μCi/ml of [^{3}H]thymidine was added during the last 2 h of hormonal treatment. [^{3}H]thymidine incorporation was measured as described in Section 2. Results represent the average ± S.D. of three independent experiments performed by quadruplicate ($n = 4$).
** $p < 0.02$ respect to control value.

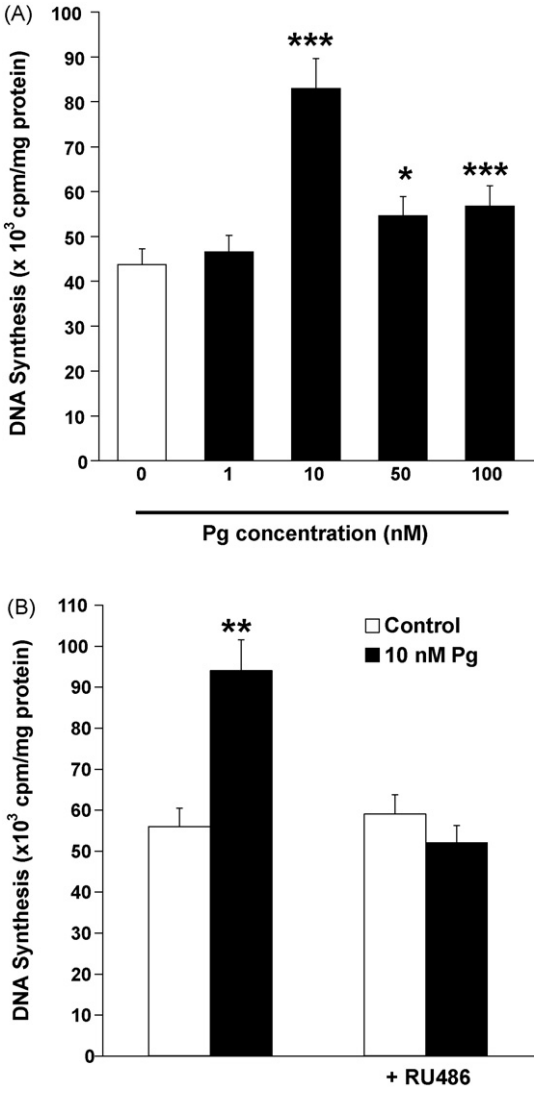

Fig. 1. Effect of progesterone on DNA synthesis. Subconfluent VSMC were incubated in serum-free medium for 24 h, and then treated with Pg in DMEM (1% FCS) at the indicated concentrations for another 24 h (panel A). In panel B, 10 nM RU486 were added to the incubation medium (fresh DMEM with 1% FCS) 1 h prior treatment with 10 nM Pg for additional 24 h. One 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 ± S.D. of three independent experiments performed in quadruplicate ($n = 4$). ***$p < 0.001$, **$p < 0.02$, *$p < 0.05$ compared with respective control value.

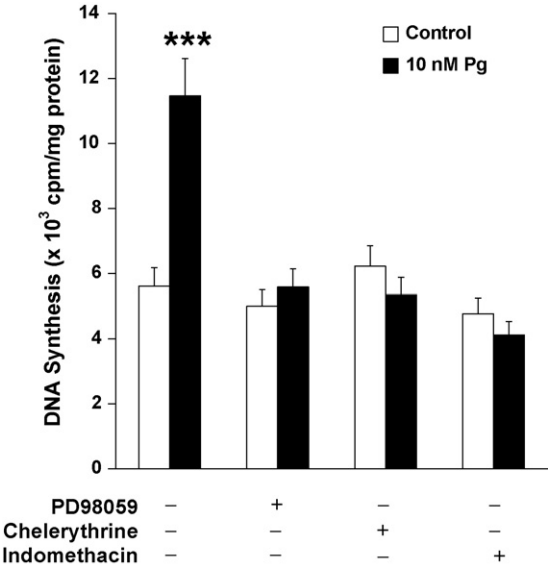

Fig. 2. Effect of PD98059, chelerythrine, and indomethacin on Pg-regulated DNA synthesis in VSMC. Subconfluent VSMC were incubated for 24 h in serum-free media. The inhibitors PD98059 (5 μM), chelerythrine (1 μM) or indomethacin (10 μM) were added to the incubation medium (fresh DMEM with 1% FCS) 1 h prior to hormonal treatment. The cells were exposed to 10 nM Pg 24 h. One 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 ± S.D. of three independent experiments performed in quadruplicate ($n = 4$). ***$p < 0.001$ respect to control value.

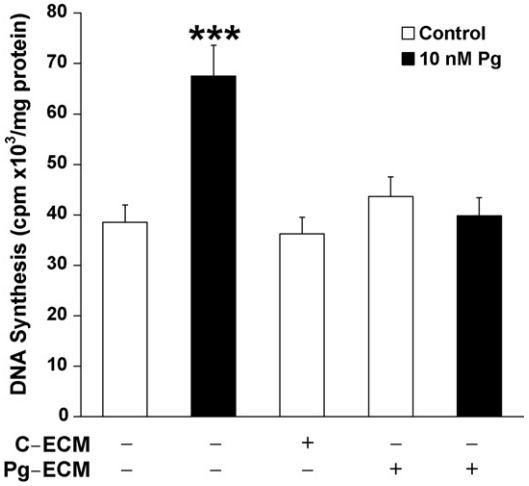

Fig. 3. Effect of conditioned EC media on Pg-regulated DNA synthesis. Subconfluent VSMC were serum starved for 24 h and further incubated with or without control EC-conditioned media (C-ECM) or Pg-conditioned media (Pg-ECM) for another 24 h. White and black bars represent the addition of vehicle or Pg to VSMC, during the last 24 h. [^{3}H]thymidine incorporation was measured as described in Section 2. Results represent the average ± S.D. of four independent experiments performed in quadruplicate ($n = 4$). ***$p < 0.001$ respect to control value.

evaluate the participation of PgR in the regulation of VSMC proliferation by Pg, we selected the compound RU486 as PgR antagonist. Fig. 1B shows that preincubation of muscle cells with 10 nM RU486 completely suppressed the hormonal action. Similar results were obtained when a wide range of RU486 concentration (10 nM to 10 μM) were tested (data not shown).

We have recently shown that Pg non-genomically activates MAPK (mitogen-activated protein kinase) and PLC/PKC (protein kinase C) signal transduction pathway and significantly enhances PKC and MAPK activities [14,15]. Upon this, we evaluated whether proliferative action of the hormone would involve these kinases systems. To that end, we selected the compounds PD98059 and chelerythrine as MAPK and PKC inhibitors respectively. In the presence of 5 μM PD98059 or 1 μM chelerythrine the stimulation of DNA synthesis elicited by 10 nM Pg was completely suppressed (Fig. 2). We also reported that the non-genomic action of Pg in vascular tissue includes cyclooxygenase activation and stimulation of PGI_2 (prostaglandin I2) synthesis [13]. Then we measured DNA synthesis in VSMC pretreated 1 h with indomethacin, a COX inhibitor. As illustrated in Fig. 2, 10 μM indomethacin blocked the proliferative effect induce by 24 h of exposure to the steroid. Altogether, these results suggest that the mitogenic action of Pg requires the hormonal activation of MAPK, PKC, and cyclooxygenase transduction pathways.

Having in mind that interactions between endothelial and VSMC are important to determine cellular behaviors, we examine whether the mitogenic response to Pg depends on these interactions. To that end, experiments using EC-conditioned media were carried out. Fig. 3 shows that Pg alone elicited one fold increase DNA synthesis, but this stimulatory effect on VSMC growth was blunted when the muscle cells were exposed to medium obtained from EC cultures. VSMC incubated with conditioned media obtained of EC treated with Pg did not stimulates [^{3}H]thymidine incorporation as compared to control muscle cells, or muscle cells incubated with conditioned media of EC exposed to vehicle alone. Furthermore, additional hormonal treatment to muscle cells incubated with Pg-treated EC-conditioned media neither affect cellular proliferation.

Since estradiol have a central role on vascular function preserving vessel integrity and preventing vascular diseases, we evaluated the effect of combined treatment with 17β-estradiol and Pg on vasoactive compound synthesis and on cellular proliferation. The fast action of both natural ovarian steroids on NO production was measured using endothelial cells exposed to 5 min treatment with 17β-E_2 or Pg added alone or simultaneously. Table 2 shows that both steroids significantly increased NO synthesis in a wide range of concentrations. When combined treatment was performed, the hormones acted in an additive manner potentiating the augmentation of NO elicited by 17β-E_2 or Pg alone. We also investigated the effect of cotreatment on the mitogenic action of Pg on VSMC. DNA synthesis was measured after 24 h treatment with the steroids alone or simultaneously. In Fig. 4 it can be observed that Pg and

Table 2
Effect of combined treatment (E2 + Pg) on nitric oxide production in endothelial cells.

	nmol NO/mg protein	% of increase above control
Control	140.2 (±17)	
10^{-10} M Pg	156.8 (±15)	12
10^{-9} M Pg	196.3 (±15)*	40
10^{-8} M Pg	256.6 (±23)**	83
10^{-7} M Pg	266.4 (±)***	90
10^{-10} M 17β-E_2	277.5 (±27)**	98
10^{-9} M 17β-E_2	309.8 (±)*	121
10^{-8} M 17β-E_2	399.6 (±12)**	185
10^{-7} M 17β-E_2	392.6 (±13)**	180
10^{-8} M Pg + 10^{-8} M 17β-E_2	555.3 (±48)***,a,b	296
10^{-7} M Pg + 10^{-7} M 17β-E_2	518.6 (±93)***,c,d	270

Starved EC were treated with Pg, E_2 or both hormones at the indicated concentrations for 5 min in fresh DMEM with 1% FCS. NO production was measured by Griess reaction as described in Section 2. Results represent the average ± S.D. of three independent experiments performed by quadruplicate ($n = 4$).

[a] $p < 0.001$ vs Pg 10^{-8} M.
[b] $p < 0.001$ vs estradiol 10^{-8} M.
[c] $p < 0.001$ vs Pg 10^{-7} M.
[d] $p < 0.001$ vs estradiol 10^{-7} M.
* $p < 0.05$ vs control.
** $p < 0.02$ vs control.
*** $p < 0.001$ vs control.

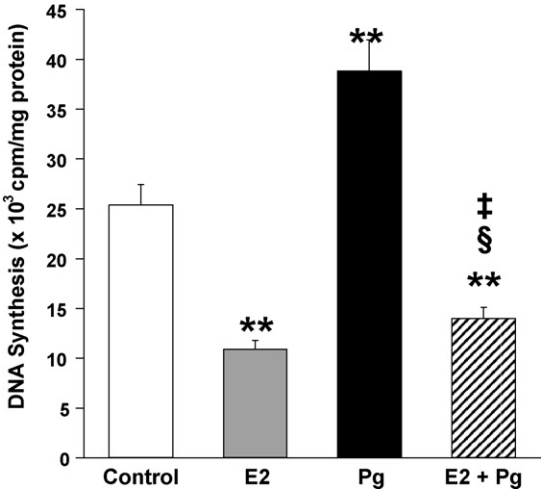

Fig. 4. Effect of combined hormonal treatment on VSMC proliferation. Subconfluent VSMC were incubated in serum-free medium for 24 h and then treated with 10 nM Pg, 10 nM E2 or both hormones for another 24 h in fresh medium with 1% FCS. One μCi/ml of [^{3}H]thymidine was added during the last 2 h of treatment. [^{3}H]thymidine incorporation was measured as described in Section 2. Results represent the average ± S.D. of three independent experiments performed in quadruplicate ($n = 4$). $^{**}p < 0.02$ respect to control value; $^{§}p < 0.001$ vs Pg alone; $^{‡}p < 0.02$ vs E2 alone.

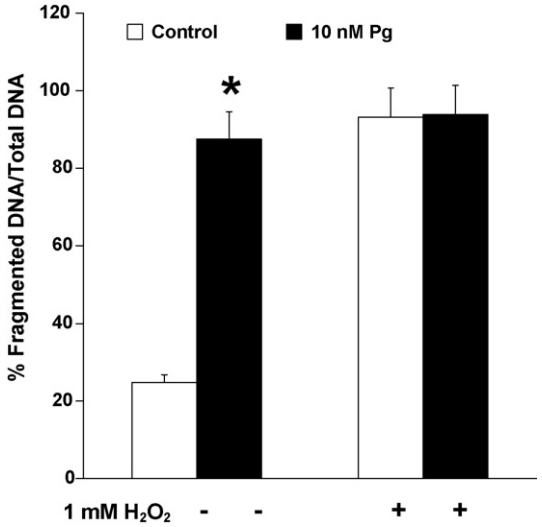

Fig. 6. Effect of Pg on vascular smooth muscle cells apoptosis. Confluent VSMC (90%) were labelled with [^{3}H]thymidine for 2 h and treated with Pg for the following 4 h. H_2O_2 was added as indicated, during the last 2 h of hormonal treatment. The amount of incorporated [^{3}H]thymidine was determined in both fractions as described in Section 2. Results represent the average ± S.D. of three independent experiments performed in quintuplicate ($n = 5$). $^{*}p < 0.05$ compared with respective control value.

17β-E_2 have opposite effects on cell proliferation. In the presence of estradiol the mitogenic action of Pg was blunted. Moreover, when both hormones were added together, DNA synthesis was significantly reduced respect to control values (13.98 ± 1.64 vs 25.37 ± 5.74 cpm × 10^3/mg protein, E_2 + Pg vs control respectively). The inhibition of DNA synthesis induced by the cotreatment was slightly lower than 17β-E_2 alone (44% vs 57% of inhibition respect to control, E_2 + Pg vs E_2, $p < 0.02$).

In order to assess the role of Pg on cell migration, wounding assays were performed. Fig. 5 shows a microphotography representative of these assays. We found that VSMC treated with 10 nM Pg induced more cell migration than non-treated cells. As can be observed, the number of cells/field in the denuded area in the monolayers exposed to the steroid was higher respect to control group.

Finally, we investigate the effect of Pg on VSMC apoptosis. To that end, we measured DNA fragmentation, a key feature of cell undergoing apoptosis. Fig. 6 shows that Pg induced programmed cell death. Four hours of treatment with 10 nM Pg enhanced DNA laddering compared to control group. The effect of Pg on cell apoptosis was slightly lower to that elicited by the apoptogen H_2O_2. Preincubation with Pg prior to H_2O_2 addition did not affect the apoptotic action of H_2O_2.

4. Discussion

The potentially beneficial or harmful effect of HRT to prevent cardiovascular disease is still controversial. Progestins represent one of the major components in this therapy. However, despite the growing knowledge of the molecular action of estrogen at vascular level, lesser is known about the effect of Pg on vascular homeostasis. Here, we provide evidence that Pg modulates growth, migration, and apoptosis of VSMC. The steroid enhances cell proliferation through a mechanism that involves the participation of PgR and MAPK, PKC, and COX pathways. The mitogenic effect of Pg was

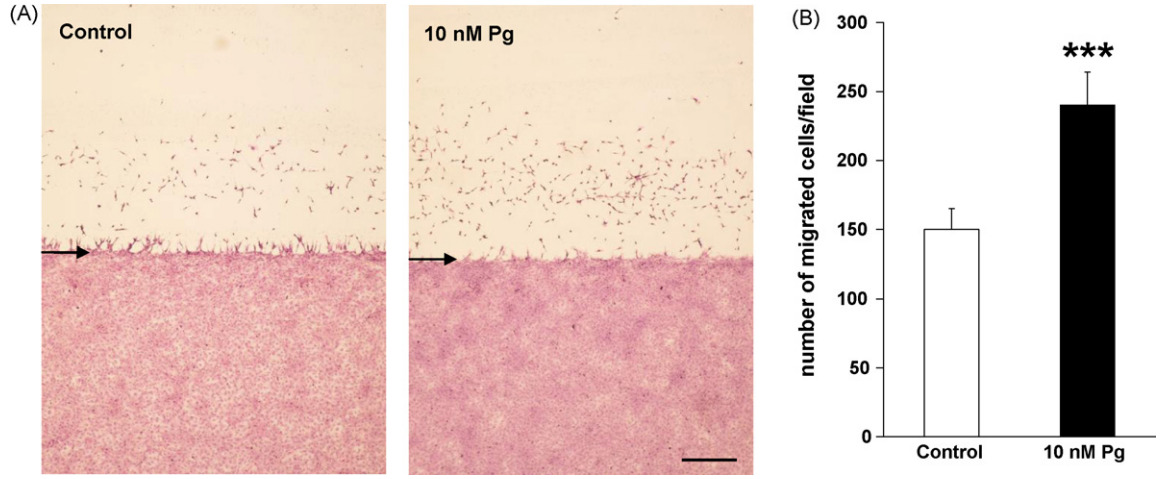

Fig. 5. Effect of Pg on vascular smooth muscle cells migration. Confluent VSMC cultured on 60-mm dishes were serum starved for 24 h. After scraping the cells besides the wound (arrows), the half remaining monolayer were treated with 10 nM Pg or vehicle for another 48 h, and then processed as described in Section 2. (A) Representative fields of each condition are shown after haematoxylin–eosin staining (magnification: 40×). The scale line represents 350 μm. (B) Bars represent the means ± S.D. of number of migrated cells/field from five separated experiments performed in quadruplicate ($n = 4$). $^{***}p < 0.001$ compared with control group.

suppressed by the presence of EC culture medium or in combined treatment with estradiol. The steroid also promotes cell migration and apoptosis

Smooth muscle cells proliferation and migration play an important role in vascular diseases. The response of vascular cell in healthy artery may not be the same as those after vascular injury. Moreover, it has been reported that Pg regulation of cell growth depends on time of treatment, the presence of mitogenic compounds or whether the assays were performed in vivo (intact blood vessels) or in vitro (isolated cells) [27]. Reports show that, although proliferation of human VSMC induced by 3% (v/v) serum or endothelin-1 can be inhibited by Pg, the hormone alone induces a significant stimulation of cell growth [28]. Our results indicated that the in vitro proliferation of female rat vascular smooth muscle cells can be stimulated by Pg. This occurs at physiological concentrations (10–100 nM), equivalent to serum levels measured in women during reproductive years [29] or equivalent to plasma levels of Pg during the 4-day rat estrous cycle [30]. We found that the mitogenic effect of Pg was observed after 24 h of treatment. Longer time intervals of treatment with Pg did not have significant effect on muscle cells proliferation. Inhibition of DNA synthesis after 48 h exposure to Pg has also been reported [27].

In the last few years, much evidence support the theory of the two step mechanism of steroid action, which involves intracellular pathways activation that converges in the synthesis of transcriptional factors required of the genomic action [31]. MAPK cascade are among the most common and important signaling pathways activated by both receptor and non-receptor tyrosine kinase. In VSMC, MAPK system control diverse cellular functions including proliferation and migration. In our experimental model the proliferative effect of Pg is dependent on MAPK activation since MEK (MAPK/ERK kinase) inhibitor PD98059 abolished the hormonal action. In addition, the presence of the PKC antagonist chelerythrine also inhibited the enhancement in DNA synthesis. It has been proposed that PKC isoforms play a central role in the regulation of vascular muscle cells survival and proliferation, events associated with the pathogenesis of atherosclerosis and hypertension [32]. Taking in account that the upstream activator of MEK (Raf) might be phosphorylated by PKC, the results obtained in this work would support the hypothesis that the mitogenic action of Pg required subsequently the activation of PKC and MAPK systems. Stimulation of VSMC proliferation by angiotensin II through activation of ERK1/2 (extracellular signal-regulated kinase 1/2) via the PKC-dependent Raf-1 pathway has been reported [33]. Eicosanoids signal transduction systems have been implicated in the regulation of arterial smooth muscle phenotype and proliferative profile [34]. ERK and COX-2 signaling pathways upregulate rat aortic muscle cells growth [35]. We showed that the mitogenic action of Pg on VSMC depends on COX pathway. Since we have previously reported that Pg non-genomically stimulates NOS, COX, MAPK, and PLC/DAG/PKC signaling pathways [13–15], our present results suggest that the mechanism of action of Pg would involve integration of genomic and non-genomic effects.

Vascular endothelium is known to strongly influence the behavior of other cells as VSMC [36]. Growth factors released from EC regulate VSMC recruitment, migration, and proliferation [37]. Functional or mechanical injury would alter the production of soluble mediators and subsequently impaired EC-VSMC interactions, promoting the progression of vascular lesions. Recent evidence shows that Pg increase VSMC proliferation through its direct action on breast cancer cells eliciting PDGF (platelet-derived growth factor) release [21]. Here we showed that the regulation of VSMC growth induced by Pg is conditioned by EC. The stimulation of DNA synthesis elicited by the steroid was blunted when the muscle cells were incubated with EC medium. This fact would be due to soluble factors or molecules that may be present in the conditioned medium, which would modulate the mechanism of action of Pg on VSMC proliferation. The identity of the putative factor/s that mediates the cross-talk between EC and VSMC on Pg signaling is under current investigation.

Progesterone, which is frequently administered concomitantly with estrogens in postmenopausal replacement therapy, is thought to antagonize some of the atheroprotective features of estrogens. This has been attributed primarily to adverse effects on lipid levels and on vascular relaxation [38,39]. However, some studies have shown that Pg did not blunt the beneficial effects of estrogens [40]. Synthetic progestins are usually used in HRT. It has been reported that Pg and medroxyprogesterone acetate (MPA) are not equivalent in terms of molecular signaling in vascular tissue. These two compounds have clearly distinct features and differently effects on estrogen signaling [41]. These controversial data display uncertainty about the vascular effects of Pg. In this study when cotreatment was performed, we obtained evidence that the natural Pg have an additive effect with estrogens, potentiating the stimulatory action of 17β-estradiol on nitric oxide synthesis. Instead of, in the genomic action on VSMC proliferation, the mitogenic effect of Pg was suppressed in the presence of estradiol. These results suggest the existence of sex hormones interactions that would involve a cross-talk between different signaling pathway activation. Overall our results suggested that the whole action of Pg on VSMC depends not only on its direct effect at cellular level, but is also conditioned by the presence of EC and other sex hormones.

We found that Pg induce VSMC apoptosis as measured by DNA fragmentation. Our evidence shows that, besides promoting cellular proliferation, Pg also causes apoptosis. In agreement with our results, several reports state that either smooth muscle cells growth or apoptosis could be induced by the same agonist. Plasma free fatty acids promote proliferation and apoptosis in VSMC through c-myc and the transcription factor E2F1 (E2F transcription factor 1) activation [25,42]. Apoptosis could be implicated to cell elimination during vascular development [43] or to induce regression of restenotic lesions or primary atherosclerotic plaque. However VSMC apoptosis could also have negative effect on the stability of atherosclerotic plaque by thinning the fibrous cap leading to plaque rupture [44].

In summary, we obtained evidence that support the hypothesis that the main cellular events involved in vascular physiology and disease, such as growth, migration, and apoptosis are suitable to be regulated by the natural sex steroid Progesterone. The mechanism of action displayed by Pg involves integration between genomic and non-genomic effects. Since our results were obtained using in vitro assays with isolated cells, the whole contribution of Pg to vascular homeostasis cannot be addressed. Vascular Pg signal must be considered as the sum of its direct action on blood cells (leukocyte, platelets), endothelium and muscle cells. Moreover, cross-talk between the biochemical action of Pg and of other vascular agonists may also occur. It is therefore difficult to draw a parallel between our results and the realistic in vivo situation. In vivo studies would provide data for a better understanding of the physiological relevance of Pg vascular action.

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