Progesterone-Induced Migration Inhibition in Male Rat Aortic Smooth Muscle Cells Through the cSrc/AKT/ERK 2/p38 Pathway-Mediated Up-Regulation of p27

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Previously, we showed that progesterone (P4) inhibits proliferation and migration of rat aortic smooth muscle cells (RASMCs). The P4-induced migration inhibition in RASMCs resulted from suppression of the Ras homolog gene family, member A (RhoA) activity mediated by cSrc activation. We also observed that P4 increased the formation of p27-RhoA complex in RASMCs. The aim of this study was to further study the involvement of p27 in P4-induced migration inhibition in RASMCs. Treatment with P4 (50 nM) increased the level of p27 protein in RASMCs. Knockdown of p27 abolished the P4-induced increases of the levels of p27 protein and decreases of cell migration in RASMCs. We conducted Western blot analyses and applied pharmacologic inhibitors to delineate the signaling pathway involved in the P4-induced p27 up-regulation and migration inhibition in RASMCs. Our data suggest that P4 increased the levels of p27 in RASMCs through activating the cSrc/AKT/ERK 2/p38 pathway mediated by non-genomic progesterone receptor. The findings of the present study highlight the molecular mechanisms underlying P4-induced migration inhibition in RASMCs. (Endocrinology 155: 1428–1435, 2014)

Cardiovascular diseases remain the leading cause of morbidity and mortality among postmenopausal women in Western society but are rare among premenopausal women (1, 2). Epidemiological studies showed that the overall incidence and mortality from almost all forms of vascular disease in premenopausal women are much lower than in men, and the incidence of cardiovascular disease in postmenopausal women gradually approaches that in age-matched men, suggesting that endogenous female sex hormones might have a protective effect against atherosclerotic vascular disease during premenopausal years (3). This hypothesis was supported by the evidence that estrogen replacement reduces the incidence of cardiovascular diseases in postmenopausal women (4, 5) and by more evidences from animal studies (6, 7). Lately, this long-standing hypothesis has been challenged by several clinical trials (8) and the benefit/risk of hormone replacement therapy is still controversial. The discrepancy might be due to the age of the women, the severity of atherosclerosis, and the regimens of hormone replacement therapy in the studies.

The effects of progesterone (P4) on cardiovascular diseases are still unclear. Previously, Grodstein et al (9) showed that the relative risk of major coronary heart disease among postmenopausal women who took estrogen with P4 was lower as compared with the risk of those who took estrogen alone. Castrated baboons receiving estrogen and P4 together had fewer vascular lesions than those receiving estrogen alone. However, some other reports indicated that the combined estrogen-progestin trial did not confirm a protective effect on cardiovascular diseases (10, 11). Although the effect of P4 on cardiovascular diseases has attracted the attention of scientists and clinicians, there is little evidence of the effect of P4 alone on atherosclerosis. Previously, our in vitro study demonstrated that P4 at physiologic levels (5–500 nM) inhibited proliferation (12, 13) and migration (14) of cultured rat aortic smooth muscle cells (RASMCs) in a concentration-dependent way.
dependent manner. In contrast to our findings, Cutini and Massheimer (15) demonstrated that P4 enhances cell proliferation, migration, and apoptosis of cultured RASMCs. The direct effect of P4 on the vascular smooth muscle cell behavior still needs to be clarified.

Atherogenesis is complex and is a degenerative process involving a variety of lesions of the arterial wall. Although the pathogenesis of atherosclerosis is not fully elucidated, one theory holds that atherosclerosis is a response of the endothelium and smooth muscle cells of the vascular wall to injury (16–19). Vascular smooth muscle cells normally reside in the media of the artery, have a low proliferative index, and are surrounded by a meshwork of several extracellular matrix components. However, in response to injury (such as injury caused by high cholesterol and high blood pressure) and to various stimuli, the activated vascular endothelium produces cytokines (such as IL-1 and TGF-β) and growth factors (such as vascular endothelial growth factor and platelet-derived growth factor-b) to promote proliferation and migration of vascular smooth muscle cells (2 key events in the formation of atherosclerotic lesion) (18). Moreover, damage to the endothelial cells also causes an inflammatory response. In the early stage of atherogenesis, the circulating monocytes adhere to the endothelium followed by their migration to the subendothelial space, and further activation into monocyte-derived macrophages, which ingest oxidized low-density lipoprotein and then turn into large foam cells contributing to the formation of the necrotic core and thinning of the fibrous cap (20).

Previously, we demonstrated that P4 inhibits proliferation (12, 13) and migration (14) of cultured RASMCs. Our data indicate that P4 at physiologic levels reduces RASMCs proliferation by increasing the levels of p21 and p27 protein and inhibits RASMCs migration through suppressing the Ras homolog gene family, member A (RhoA) activity (14). In the present study, the signaling pathway involved in the P4-induced migration inhibition in RASMCs was further investigated.

Materials and Methods

Cell cultures

Rat aortic smooth muscle cells (RASMCs) harvested from the thoracic aortas of adult male Sprague Dawley rats (200–500 g) by enzymatic dissociation were grown in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO), penicillin (100 U/mL), and streptomycin (100 mM/mL, GIBCO) in a humidified 37°C and 5% CO2 incubator. For the experiments, the cells were treated with either P4 (50 nM) in 0.05% dimethylsulfoxide (Sigma-Aldrich) for the indicated times or 0.05% dimethylsulfoxide (control).

Reagents

Antibodies against p-AKT, AKT, p-ERK, p-p38, p38, poly (ADP-ribose) polymerase, p65, p-IkBα, or IkBα were purchased from Santa Cruz Biotechnology, Inc. Anti-α-tubulin, anti-ERK, and anti-p27 antibody were purchased from Sigma-Aldrich. Anti-p-cSrc antibody was purchased from Cell Signaling Technology, Inc. Anti-glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Jackson ImmunoResearch Laboratories. Anti-cSrc antibody was purchased from Abcam. Anti-progestrone receptor (PR) antibody was purchased from NeoMarkers. 4-Amino-5-(4-chlorophenyl)-7-(dimethylmethoxy)pyrazolo[3,4-d] pyrimidine (PP2) was purchased from A.G. Scientific Inc. Wortmannin, U0126, SB203580, and Bay 11-7082 were purchased from Cayman Chemicals. Org 31701 was kindly provided by Merck Sharp & Dohme Corp.

Wound-healing assay

Wound healing assay was performed as previously described (21). Briefly, cells were grown in 24-well plates. White tips for a 10-μL micropipette were used to scalp a “wound” in a cell monolayer. The images captured at the beginning were compared with the images after 18 hours in a humidified 37°C, CO2 incubator. The cells migrating to close the wound were counted to quantify the migration rate of the cells.

Western blot analysis

To determine the protein levels in RASMCs, the total proteins were extracted and Western blot analyses were performed as described previously (13). Electrophoresis was carried out using a 12% sodium dodecyl sulfate-polyacrylamide gel (50 μg protein per lane). Separated proteins were transferred onto polyvinylidene difluoride membranes, treated with 1% BSA/0.02% NaNO3 to block the nonspecific IgGs, incubated for 1 hour with specific primary antibody (0.2 μg/mL), and then incubated with second antibody (Jackson ImmunoResearch Laboratories) linked to horseradish peroxidase (1:10 000) for 1 hour. Subsequently, the blot was developed using the enhanced chemiluminescence system (Amer sham). The intensity of each band was quantified by densitometry analysis using Image Pro-Plus 4.5 Software.

Small interfering RNA knockdown assay

Expression of p27 was knocked down in RASMCs with at least 3 independent small interfering RNAs (siRNAs). The target sequences of p27 mRNA were selected to suppress p27 gene expression. Nontarget sequences of each siRNA were used as controls (21). After BLAST analysis to verify that there were no significant sequence homologies with other human genes, the selected sequences were inserted into BglII/HindIII-digested pSUPER vectors to generate the pSUPER-Si p27 (p27-Si) and pSUPER-nontarget siRNA. The cells were transfected with either combined three antisense siRNAs or nontarget RNA (control). Three different antisense siRNAs targeted against different parts of the p27 sequence are listed below: Si-1: 5′-GATCCCC CCGAAGCGACCTGCAAGCCGTAATCCCAAGAGATCTTCAGTGGTGTC AGGTCGTTTCTTTTTTA-3′; Si-2: 5′-GATCCCACACCCGGAGAAAGATGTCCATCAAGAGATCTTCAGTGGTGTC AGGTCGTTTCTTTTTTA-3′; Si-3: 5′-GATCCCCGAAGCCGACCTGCAAGCCGTAATCCCAAGAGATCTTCAGTGGTGTC AGGTCGTTTCTTTTTTA-3′; NT: 5′-GATCCCCGAAGCCGACCTGCAAGCCGTAATCCCAAGAGATCTTCAGTGGTGTC AGGTCGTTTCTTTTTTA-3′.
All constructs were confirmed by DNA sequence analysis. The transfection protocol has been previously described (22). Briefly, \(2 \times 10^5\) cells were washed twice with PBS and mixed with 0.5 μg of plasmid, and then one electric pulse was applied for 20 msec under a fixed voltage of 1.3 kV on a pipette-type MP-100 micro-porator (Digital Bio).

**Cell transfection**

For transient transfection of the indicated constructs into RASMCs, jetPEI-RASMCs transfection reagent (Polyplus Transfection) was used, and the transfection was performed as previously described (23, 24).

**Nuclear extraction**

To examine the effect of P4 on nuclear translocation of nuclear factor-κB (NF-κB) (p65), the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) were used, and the extraction was performed as previously described (23).

**Statistical analysis**

All data were expressed as the mean value ± SEM. Comparisons were subjected to ANOVA followed by Fisher’s least significant difference test. Significance was accepted at \(P < .05\).

## Results

### Involvement of p27 protein on the P4-induced migration inhibition in RASMCs

Since we previously demonstrated that P4 at physiologic levels (5–500 nM) inhibited migration of cultured RASMCs in a concentration-dependent manner (14), we further studied the involvement of p27 protein on the P4 (50 nM)-induced migration inhibition in RASMCs. As illustrated in the Figure 1a, P4 (50 nM) time-dependently increased the levels of p27 protein in RASMCs. To investigate whether the increased p27 is involved in the P4-induced migration inhibition in RASMCs, we knocked down the p27 expression using the siRNA technique. As shown in Figure 1b, transfection of RASMCs with p27-siRNA reduced the P4-induced increases of the level of p27 protein. Figure 1c shows that transfection with p27-siRNA, but not nontarget siRNA, abolished the P4-induced migration inhibition in RASMCs.

### Candidate molecules involved in the P4-induced migration inhibition in RASMCs

To delineate the signaling pathway involved in the P4-induced up-regulation of p27 and migration inhibition in

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**Figure 1.** Involvement of p27 on the P4-induced migration inhibition in RASMCs. P4 (50 nM) increased the protein levels of p27 in RASMCs in a time-dependent manner. Top panel shows Western blot analysis; lower panel shows a graph of quantitation of these data adjusted with α-tubulin protein level and expressed as ratio over control. Values represent the means ± SE (n = 3). *, \(P < .05\) different from control group (a). Transfection of RASMCs with p27 siRNA followed by P4 (50 nM) treatment for 13 hours. Treatment with p27 siRNA reduced the P4-induced increases of p27 expression in RASMCs. Values shown in parentheses represent the quantified results (b). The P4-induced migration inhibition in RASMCs was abolished by transfection with p27 siRNA, but not nontarget RNA. Values represent the means ± SE (n = 3) (c). *, \(P < .05\) different from control group. Con, control; NT-siRNA, nontarget RNA.
RASMCs, we initially examined the changes of the activity of several candidate molecules, which have been indicated to be involved in regulation of cell migration, in P4-treated RASMCs. Because the molecules of cSrc (25), AKT (26), ERK (27), and p38 (28) have been suggested to play important roles in the regulation of vascular smooth muscle cell migration, we examined the effect of P4 treatment on the activity of these candidate molecules. Treatment with P4 (50 nM) increased the levels of p-cSrc (Supplemental Figure 1a published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org, and Figure 2a), p-AKT (Supplemental Figure 1b and Figure 2b), p-ERK1/2 (Supplemental Figure 1c and Figure 2c), and p-p38 (Supplemental Figure 1d and Figure 2d) in RASMCs at 10–20 seconds after treatment. Pretreatment with a PR-specific antagonist, Org 31710 (1 μM), abolished the P4-induced increases of p-cSrc (Supplemental Figure 2a and Figure 3a), p-AKT (Supplemental Figure 2b and Figure 3b), p-ERK1/2 (Supplemental Figure 2c and Figure 3c), and p-p38 (Supplemental Figure 2d and Figure 3d), suggesting that activation of these molecules might be involved in the P4-induced up-regulation of p27 and migration inhibition in RASMCs and mediated by PR activation.

**Signaling pathway involved in the P4-induced up-regulation of p27 and migration inhibition in RASMCs**

Previously, we demonstrated that PR and cSrc forms the complex and the formation of p-cSrc-PR complex was increased by P4 treatment (14), suggesting that cSrc is the upstream molecule in regulating the P4-induced migration inhibition. As shown in Figure 4a, pretreatment with a cSrc inhibitor, PP2 (200 nM), abolished the P4-induced increases of p-cSrc (Figure 2a), p-AKT (Figure 2b), p-p38 (Figure 2c), and p-ERK1/2 (Figure 2d), and decreases of the migration activity (Figure 2e), suggesting that cSrc is the most upstream molecule involved in regulating the P4-induced migration inhibition. Pretreatment of RASMCs with a phosphatidylinositol 3-kinase inhibitor, wortmannin (100 nM), abolished the P4-induced increases of p-AKT (Figure 3a), p-p38 (Figure 3b), and p-ERK1/2 (Figure 3c), suggesting that AKT is the upstream molecule of ERK.

![Figure 2](https://academic.oup.com/endo/article-abstract/155/4/1428/2423350)

**Figure 2.** cSrc is the upstream molecule involved in the P4-induced migration inhibition in RASMCs. Pretreatment of RASMCs with a cSrc antagonist, PP2 (200 nM), abolished the P4 (50 nM)-induced activation of cSrc (a), AKT (b), p38 (c), and ERK1/2 (d), and migration inhibition (e). Quantitative results of phosphorylated proteins were shown after being adjusted with their own total protein level and expressed as fold induction of control (a–d). Values represent the means ± SE (n = 3). *P < .05 different from control group. Con, control.
and p38. Moreover, pretreatment of the cell with a mitogen-activated protein/extracellular signal-regulated kinase inhibitor, U0126 (1 μM), abolished the P4-induced increases of p-ERK1/2 (Figure 4a) and p-p38 (Figure 4b), whereas a p38 inhibitor, SB 203580 (1 μM), abolished the P4-induced increases of p-p38 (Figure 4c), but did not affect the levels of p-ERK1/2 (Figure 4d), suggesting that p38 is the downstream molecule of ERK1/2 or parallel to ERK1/2. To clarify this issue, the experiment illustrated in Figure 4e was conducted. Pretransfection of RASMC with dominant-negative ERK 2 cDNA abolished the P4-induced increases of p-p38 (Figure 4e), suggesting that p38 is the downstream molecule of ERK 2. To further confirm that these molecules are involved in the P4-induced increases of the levels of p27 protein, the specific inhibitors were applied to examine their effects on the P4-induced up-regulation of p27. The P4-induced increases of the levels of p27 were abolished by pretreatment of the cell with PP2 (Figure 5a), SB 203580 (Figure 5b), U0126 (Figure 5c), or wortmannin (Figure 5d).

We previously demonstrated that P4 increased p27 expression through NF-κB (p65)-induced up-regulation of p53 in HUVEC (23). Accordingly, we further examined the involvement of NF-κB activation in the P4-induced up-regulation of p27 in RASMCs. As illustrated in Figure 6a, the translocation of p65 was increased after P4 treatment. Moreover, pretreatment of RASMCs with a selective IκBα phosphorylation inhibitor, Bay 11-7082 (10 nM), blocked the P4-induced up-regulation of p27 (Figure 6b). The P4-induced p65 nuclear translocation and IκBα phosphorylation were blocked by treatment of the cell with 1 μM of a p38 inhibitor, SB 203580 (Figure 6c). The entire gel pictures of Figures 1–6 have been shown in Supplemental Figures 3–5.

**Discussion**

Previously, we demonstrated that P4 at physiologic levels (5–500 nM) inhibited migration of cultured RASMCs...
in a concentration-dependent manner through the cSrc signaling pathway-mediated inactivation of RhoA. This effect is abolished by pretreatment with a PR antagonist, RU486. Here, we further demonstrated that the PR/cSrc/AKT/ERK/p38-mediated up-regulation of p27 is involved in the P4-induced migration inhibition in RASMCs.

P27, an inhibitor of cyclin/cyclin-dependent kinase (CDK) complexes, is known to play a crucial role in cell cycle regulation. It can bind with cyclin-CDK complexes and inactivate specific CDK enzymes required for cell di-

Figure 5. Activation of the cSrc/AKT/ERK1/2/p38 pathway is involved in the P4-induced increases of p27 in RASMCs. Pretreatment of RASMCs with 200 nM PP2 (a), 1 μM SB203580 (b), 1 μM U0126 (c), or 100 nM wortmannin (d) abolished the P4-induced up-regulation of p27. The electrophoresis membrane was probed with α-tubulin antibody to verify equivalent sample loading. Con, control; SB, SB 203580; WM, wortmannin.

vision and thereby arrest the cell cycle in the G1 phase (29, 30). P27 also functions as a tumor suppressor in a variety of different cancers (31). In addition to the cell cycle regulation activity, p27 has been demonstrated to be involved in regulation of cancer cell differentiation (32, 33) and apoptosis (34) and cell migration (35). Here, we showed that elevation of p27 is involved in the P4-induced migration inhibition in RASMCs. P4 at a concentration of 50 nM increased the levels of p27 in RASMCs. Knockdown of p27 abolished the P4-induced migration inhibition, suggesting that up-regulation of p27 contributed to the P4-induced migration inhibition in RASMCs.

We previously demonstrated that P4 inhibited endothelial cell proliferation through activation of cSrc (23). cSrc can form the complex with PR in the absence of P4 treatment. However, P4 can increase the formation of cSrc-PR and p-cSrc-PR complexes. The P4-induced inhibition of endothelial cell proliferation is mediated by PR activation of extranuclear signaling pathways. In the present study, we examined whether cSrc activation is also involved in the P4-induced migration inhibition in RASMCs. The level of p-cSrc in RASMCs was significantly increased at 10 seconds after P4 treatment. Pretreatment of RASMCs with a cSrc inhibitor, PP2 (200 nM), abolished the P4-induced increases of p27 protein and migration inhibition, suggesting that cSrc is the upstream molecule in regulating P4-induced increases of p27. This notion was supported by the evidence that pretreatment of RASMCs with PP2 blocked the P4-induced activations of AKT, p38, and ERK1/2. The P4-induced activations of p38 and ERK1/2 were also abolished by a phosphatidylinositol 3-kinase inhibitor, wortmannin, suggesting that AKT is the upstream molecule of p38 and ERK1/2. Pretreatment of RASMCs with a mitogen-activated protein/ex-

Figure 6. Involvement of NF-κB activation on the P4-induced up-regulation of p27 in RASMCs. Treatment of RASMCs with P4 (50 nM) for 60 seconds increased the level of p-IκBα and p65 nuclear translocation. PARP and G3PDH were used as a nuclear and cytosolic protein marker, respectively, to confirm the purities of isolation and to verify equivalent sample loading (a). Pretreatment with Bay 11-7082 (10 nM), the selective IκBα phosphorylation inhibitor, abolished the P4-induced up-regulation of p27 in RASMCs. The electrophoresis membrane was probed with α-tubulin antibody to verify equivalent sample loading. Quantitative results of p27 were shown after adjusted with α-tubulin protein level and expressed as fold induction of control. Values represent the means ± SE (n = 3). *, P < .05 different from control group (b). Pretreatment with SB 203580 (1 μM) blocked the P4-induced IκBα phosphorylation and p65 nuclear translocation (c). BAY, Bay 11-7082; Con, control; G3PD, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly (ADP-ribose) polymerase; SB, SB 203580.
tracellular signal-regulated kinase kinase inhibitor, U0126, blocked the P4-induced activations of ERK1/2 and p38, whereas a p38 inhibitor, SB203580, blocked the P4-induced activation of p38, but not ERK1/2. Furthermore, pretransfection of RASMC with dominant-negative ERK 2 cDNA abolished the P4-induced increases of p-p38. These data suggest that p38 is the downstream molecule of ERK 2. Moreover, PP2, SB203580, U0126, and wortmannin abolished the P4-induced up-regulation of p27. Taken together, these data suggest that P4 induced p27 up-regulation through the PR/cSrc/AKT/ERK 2/p38-mediated signaling pathway.

It has been demonstrated that p38 regulates transcriptional activity of NF-κB in primary human astrocytes through acetylation of p65 (36). In unstimulated cells, the NFκB dimers are sequestered in the cytoplasm by its inhibitory protein, IκBα. Phosphorylation of IκBα results in dissociation of NFκB from the NFκB-IκB complex and leads to nuclear translocation of NF-κB (37–40). In human vascular endothelial cells, we previously demonstrated that the cSrc/Kras/Raf-1/ERK 2/NF-κB signaling pathway contributes to the P4-induced up-regulation of p53 (23), and subsequently increases the levels of p27 and p21. In the present study, our data showed that P4 induced IκBα phosphorylation and nuclear translocation of p65. Pretreatment with a p38 inhibitor, SB 203580, blocked the P4-induced IκBα phosphorylation, p65 nuclear translocation, and p27 up-regulation. Furthermore, a selective IκBα phosphorylation inhibitor, Bay 11-7082, also blocked the P4-induced up-regulation of p27. Taken together, these data suggest that activation of NF-κB is involved in the P4-induced up-regulation of p27.

Previously, Cutini and Massheimer (15) demonstrated that P4 enhances cell proliferation, migration, and apoptosis of RASMCs. In contrast to their report, our results from the previous and present studies showed that P4 inhibited cell proliferation and migration of RASMCs (12–14). The discrepancy might be due to the regimens of P4 treatment in these 2 studies. In their study, RASMCs were treated with the medium containing 1% FBS, whereas we treated the cells with the medium containing 10% FBS. RASMCs proliferate and migrate very slowly in the medium containing 1% FBS. Although Cutini and Massheimer showed a significant enhancement of DNA synthesis at P4 treatment for 24 hours but not 48–96 hours, the value of [3H]thymidine incorporation is very low, and it is not clear whether the proliferation of RASMCs was affected significantly by P4 treatment in their study. Moreover, the cells were starved for 24 hours in serum-free medium and then treated with P4 or vehicle for additional 48 hours in their migration assay. These treatments result in a very scattered distribution and few numbers of the migrated cells. This might explain why our results are contrary to those reported by Cutini and Massheimer.

In conclusion, the results from the present in vitro study indicate that P4 directly inhibited RASMCs migration through the cSrc/AKT/ERK 2/p38 pathway-mediated up-regulation of p27. The molecular mechanism underlying p27-induced RhoA inactivation is still under investigation.

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