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Previously we demonstrated that progesterone at physiologic levels dose dependently inhibited cell proliferation in cultured rat aortic smooth muscle cells (RASMCs). However, the molecular mechanism underlying of progesterone-induced antiproliferation was not clear. Here we demonstrated that progesterone induced a reduction of the [^{3}H]thymidine incorporation into RASMCs during the S-phase of the cell cycle. Western blotting analysis revealed that the protein levels of cyclin A, cyclin E, and cyclin-dependent-kinase (CDK) 2 but not cyclin D1 and CDK4 decreased after progesterone treatment, but those of CDK-inhibitory proteins, p21 and p27, increased. Immunoprecipitation showed that the formations of the CDK2-p21 and CDK2-p27 complex were increased and the assayable CDK2 kinase activity was decreased in the progesterone-treated RASMCs. In contrast, the formations of the CDK4-p21 and CDK4-p27 complex and the assayable CDK4 kinase activity were not changed significantly by progesterone treatment. Pretreatment of RASMCs with a p21 or p27 antisense oligonucleotide reduced the progesterone-induced inhibition of [^{3}H]thymidine incorporation into RASMCs. In conclusion, these data suggest that progesterone inhibits RASMCs proliferation by increasing the levels of p21 and p27 protein, which in turn inhibit CDK2 kinase activity, and finally interrupt the cell cycle. (*Endocrinology* 144: 2785–2790, 2003)

ATHEROSCLEROSIS AND ITS complications, such as coronary artery disease, stroke, and peripheral vascular disease, remain the principal causes of death in developed countries. Although the pathogenesis of atherosclerosis is not fully elucidated, one theory holds that atherosclerosis is a response of the vascular wall to injury (1–4). In response to injury and various stimuli, the activated vascular endothelium produces cytokines and growth factors to promote the growth and migration of vascular smooth muscle cells, key events in the formation of atherosclerotic lesions in humans. Thus, one of the most important goals in the study of prevention of atherosclerosis is to identify factors that inhibit vascular smooth muscle cell proliferation and migration.

In humans, the epidemiological studies showed that premenopausal women have a much lower mortality from atherosclerotic cardiovascular disease than men, suggesting that sex hormones might have a cardioprotective effect (5). This hypothesis was supported by the evidence that estrogen replacement reduces the incidence of cardiovascular diseases in postmenopausal women (6). In experimental animals, estrogen administration inhibits the development of experimentally induced atherosclerosis (7–10). There has been little information on the effects of progesterone on cardiovascular disease. Previously, Grodstein and Stamper (11) showed that the relative risk of major coronary heart disease among postmenopausal women who took estrogen with progesterone was 0.39, compared with the risk of those who took estrogen alone, which was 0.60 (in which the risk among women who took no hormones was set at 1.0). In castrated baboon receiving estradiol and progesterone together had fewer vascular lesions than those receiving estradiol alone.

The exact mechanisms of the cardioprotective or atheroprotective effect of sex hormones are not well understood. Smooth muscle cell proliferation and migration play an important role in the genesis of the atherosclerotic plaque. 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 the process of atherogenesis, smooth muscle cell proliferation is increased in the forming neointima and innermost part of the underlying media (12). Previous studies have demonstrated that estrogen exerts a direct effect to cause vasodilation, suppress collagen synthesis, and act as calcium antagonist properties. Estrogen also exerts an indirect effect to inhibit low-density lipoprotein oxidation, attenuate coronary and aortic low-density lipoprotein accumulation, and increase plasma high-density lipoprotein levels (13). However, there is little evidence of a direct effect of sex hormones on proliferation of vascular smooth muscle cells. The localization of estrogen and progesterone receptors in the medial layer of aorta led us to hypothesize that sex hormones might have a direct effect on the proliferation of the vascular smooth muscle cell.

Previously we demonstrated that progesterone (the natural hormone), but not estrogen, at physiologic levels inhibited DNA synthesis and decreased cell number in cultured rat and human aortic smooth muscle cells in a dose-dependent manner (14). However, the underlying mechanism of progesterone-induced antiproliferation was not clear. The findings of this study will provide important insights into the molecular and cellular mechanisms of atheroprotective ef-

Abbreviations: AS, Antisense oligonucleotide; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; DTT, dithiothreitol; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; G3PDH, glycerol-3-phosphate dehydrogenase; RASMC, rat aortic smooth muscle cell.

fects of progesterone. Only when the mechanism of atheroprotective effects of progesterone is fully understood can we begin to design a strategy for preventing and treating atherosclerosis and its complications.

Materials and Methods

Materials

Water-soluble progesterone was purchased from Sigma (St. Louis, MO). Dithiothreitol (DTT), HEPES, EDTA, glycerol, phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, sodium dodecyl sulfate (SDS), Nonidet P-40, trypsin-EDTA, and kanamycin were purchased from Life Technologies, Inc. (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT). Antibodies specific for cyclins, cyclin-dependent-kinases (CDKs), and CDK inhibitors (CKIs) were purchased from Transduction Laboratories, Inc. (Lexington, KY). An antibody specific for glycerol-3-phosphate dehydrogenase (G3PDH) was purchased from Biogenesis (Kingston, NH). Antimouse IgG-conjugated alkaline phosphatase was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). 4-Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Protein assay agents were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

Cell culture

Rat aortic smooth muscle cells (RASMCs) were harvested from the thoracic aortas of adult male Sprague Dawley rats (200–250 g) by enzymatic dissociation. The cells were grown in DMEM supplemented with 10% FBS and penicillin (100 U ml^{-1}), streptomycin (100 μg/ml), and 25 mM HEPES (pH 7.4) in a humidified 37 C incubator. After the cells had grown to confluence, they were disaggregated in trypsin solution, washed with DMEM containing 10% FBS, centrifuged at 125 $\times$ *g* for 5 min, resuspended, and then subcultured according to standard protocols. Cells from passages 5–9 were used.

[^{3}H]Thymidine incorporation

As previously described (15, 16), RASMCs at a density of 1×10^4 cells/cm^3 were applied to 24-well plates in growth medium (DMEM plus 10% FBS). After the cells had grown to 70–80% confluence, they were rendered quiescent by incubation for 72 h in DMEM containing 0.04% charcoal/dextran-treated FBS (Hyclone Laboratories, Inc.). Phenol red-free DMEM was used in the experiments with progesterone. Water-soluble progesterone or PBS in 2% FBS was added to the cells at various concentrations, and the mixture was allowed to incubate for 24 h. During the last 2 h of the incubation with or without progesterone, [^{3}H]thymidine was added at 1 μCi/ml^{-1} (1 μCi = 37 kBq). Incorporated [^{3}H]thymidine was extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

Protein preparation and Western blotting

To determine the expression levels of cyclins, CDKs, CKIs, and G3PDH in RASMCs, the total proteins were extracted, and Western blot analyses were performed as described previously (16). Briefly, RASMCs were cultured in 15-cm Petri dishes. After reaching subconfluence, the cells were rendered quiescent and then treated with various concentrations of progesterone for 24 h and incubated in a humidified incubator at 37 C. After incubation, the cells were washed with PBS (pH 7.4), incubated with extraction buffer (10 mM Tris, pH 7.0; 140 mM NaCl; 2 mM phenylmethylsulphonyl fluoride; 5 mM DTT; 0.5% Nonidet P-40; 0.05 mM pepstatin A; and 0.2 mM leupeptin) with gentle shaking and then centrifuged at 12,500 $\times$ *g* for 30 min. The cell extract was then boiled in a ratio of 1:1 with sample buffer (100 mM Tris, pH 6.8; 20% glycerol; 4% SDS; and 0.2% bromophenol blue). Electrophoresis was performed using 10% SDS-polyacrylamide gel (2 h, 110 V, 40 mA, 50 μg protein per lane). Separated proteins were transferred to polyvinyl difluoride membranes (3 h, 40 V), treated with 5% fat-free milk powder to block the nonspecific IgGs, and incubated for 1 h with specific antibody for cyclins, CDKs, CKIs, or G3PDH. The blot was then incubated with antimouse or antirabbit IgG linked to alkaline phosphatase (1:1000) for 1 h. Subsequently the membrane was developed with 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate as a substrate.

Immunoprecipitation

As previously described (16), CDK2 or CDK4 was immunoprecipitated from 200 μg protein by using anti-CDK2 or anti-CDK4 antibody (2 μg) and protein A agarose beads (20 μl). The precipitates were washed five times with lysis buffer and once with PBS. The pellet was then resuspended in sample buffer (50 mM Tris, pH 6.8; 100 mM bromophenol blue; and 10% glycerol) and incubated at 90 C for 10 min before electrophoresis to release the proteins from the beads.

CDK assay

As previously described (17), CDK2 or CDK4 immunoprecipitates from progesterone-treated and control RASMCs were washed three times with lysis buffer and twice with kinase assay buffer (50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; and 1 mM DTT). Phosphorylation of histone H1 (for CDK2) and glutathione-*S*-transferase fusion protein (for CDK4) were measured by incubating the beads with 40 μl hot kinase solution [0.25 μl (2.5 μg) histone H1, 0.5 μl (γ-^{32}P) ATP, 0.5 μl 0.1 mM ATP, and 38.75 μl kinase buffer] at 37 C for 30 min. The reaction was stopped by boiling the sample in SDS sample buffer for 5 min, and the products were analyzed by 10% SDS-PAGE. The gel was dried and visualized by autoradiography.

Flow cytometry

As previously described (18), the cells were seeded onto 100-mm dishes and grown in DMEM supplemented with 10% FBS. After the cells had grown to subconfluence, they were rendered quiescent and challenged with 10% FBS. Then after release using trypsin-EDTA, they were harvested at various times, washed twice with PBS/0.1% dextrose, and fixed in 70% ethanol at 4 C. Nuclear DNA was stained with a reagent containing propidium iodine (50 mg ml^{-1}) and DNase-free RNase (2 U ml^{-1}) and measured using a fluorescence-activated cell sorter (FACS). The proportion of nuclei in each phase of the cell cycle was determined using established CellFIT DNA analysis software (Becton Dickinson and Co., San Jose, CA).

Statistical analysis

Values represent the means ± SEM. Three to six samples were analyzed in each experiment. Comparisons were subjected to one-way ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

Results

Arrest of cell cycle in G0/G1

Previously we demonstrated that progesterone inhibited DNA synthesis and decreased cell number in cultured RASMCs in a dose-dependent manner (14). To further study the actions of progesterone on the cell cycle, the cells were switched to media with 0.04% FBS for 72 h to render them quiescent and synchronize their cell cycle activities. Then they were returned to media with 10% FBS and, at various times thereafter, they were treated with [^{3}H]thymidine. Figure 1A shows a reduction of the thymidine incorporation into RASMCs during the S-phase of the cell cycle. Figure 1B shows the FACS analyses of DNA content at 24 h after release from quiescence by incubation in culture media supplemented with 10% FBS and PBS or progesterone (500 nM) in PBS. The data reveal that progesterone induced a significant accumulation of cells at the G0/G1 phase of the cell cycle, suggesting that the observed growth inhibition effect of pro-

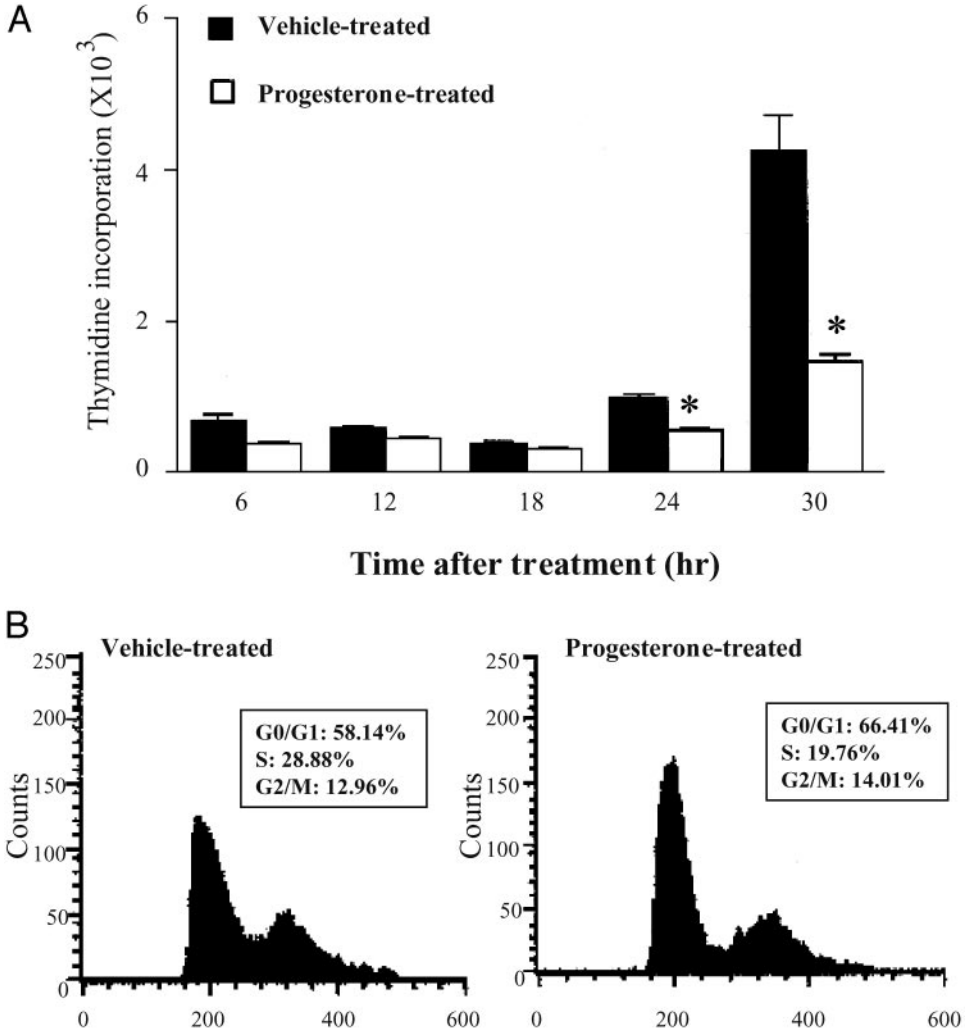

FIG. 1. Time-dependent inhibition of cell cycle in RASMCs by progesterone. To study the time-dependent progesterone on the cell cycle, [^{3}H]thymidine incorporation was conducted after RASMC release from quiescence by incubation in culture media supplemented with 10% FBS and PBS (control) or 500 nM progesterone in PBS (A). Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *, Progesterone-treated group different from PBS-treated group (n = 6). FACS analysis of DNA content was performed after 24-h release from quiescence by incubation in culture media supplemented with 10% FBS and PBS without (control) or with 500 nM progesterone (B). Percentage of cells at the G0/G1, S, or G2/M phase of the cell cycle was determined using established CellFIT DNA analysis software. Four samples were analyzed in each group, and values represent the mean ± SEM.

gesterone was due to a retardation of DNA replication, thereby inhibiting further progress in the cell cycle.

Alterations in cell cycle activity

It has been generally believed that coordinated successive activation of certain CDKs occurs late in the G1 phase and is instrumental in the transition from the G1 to the S-phase. This CDK activation is in turn modulated by association with a number of regulatory subunits called cyclins and a group of CDK-inhibitory proteins designated CKIs. Cyclins have been identified as cyclins A, D1, D3, and E. Using Northern blot analysis, we previously demonstrated that progesterone dose dependently inhibits the expression levels of cyclin A and E mRNA but not cyclin B and D1 mRNA (14). As illustrated in Fig. 2, Western blot analysis demonstrated that progesterone dose dependently inhibited the protein levels of cyclin A and E but not cyclin D1. We also examined the changes of CDK levels in the progesterone-treated RASMCs. In response to progesterone treatment, the levels of CDK2, but not CDK4, protein were decreased in a dose-dependent manner (Fig. 2). Because not only the protein levels of cyclins and CDKs but also a group of CKIs can control the CDK activity, we examined the protein levels of p21 and p27, two known CKIs, in the progesterone-treated RASMCs. Figure 3A shows that the protein levels of p21 and p27 were increased in the progesterone-treated RASMCs, compared with the PBS-treated cells (control). We further conducted an immunoprecipitation assay to examine the effect of progesterone on the formation of CDK-CKI complex. In progesterone-treated cells, the formations of the CDK2-p21 and CDK2-p27 complex, but not CDK4-p21 and CDK4-p27 complex, were increased (Fig. 3B), and the assayable CDK2 kinase activity, but not CDK4 activity, was decreased (Fig. 3C). These changes are in a dose-dependent manner. These findings suggest that progesterone induced an inhibition of CDK2 activity and led to the impairment of RASMCs in the transition from G1 to S-phases.

P21 and p27 are the key regulators for the progesterone-induced G0/G1 arrest

As illustrated in Fig. 3, A and B, the levels of p21 and p27 protein and formations of CDK2-p21 and CDK2-p27 complex were dose dependently increased in progesterone-treated RASMCs, suggesting that up-regulation of p21 and p27 might be responsible for the progesterone-mediated G0/G1 arrest in these cells. To further demonstrate that in the

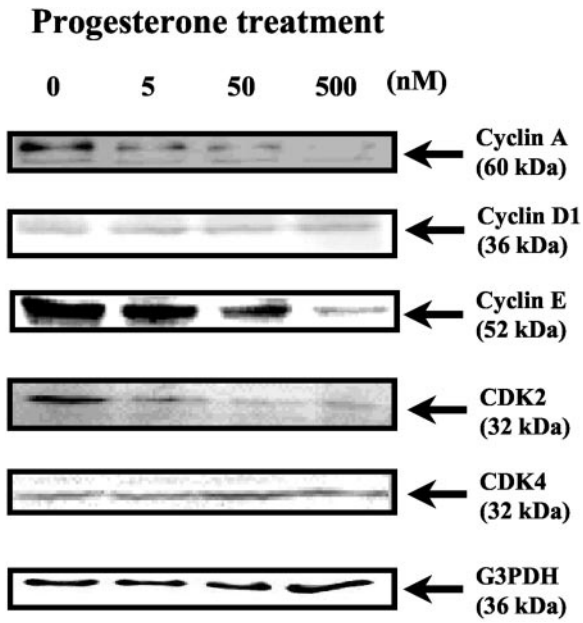

FIG. 2. Effect of progesterone on the protein levels of cyclins and CDKs. Proteins were extracted from the cultured RASMCs at 24 h after progesterone treatment and probed with proper dilutions of specific antibodies. Progesterone dose dependently decreased the levels of cyclin A and cyclin E protein as well as CDK2 protein but not cyclin D1 and CDK4 protein. Membrane was probed with anti-G3PDH antibody to verify equivalent loading.

progesterone-treated RASMCs, increased p21 and p27 expressions correlated with G0/G1 arrest, the experiment illustrated in Fig. 4 was carried out. Thus, in the sample labeled P4 for progesterone (500 nM) treated alone, the [^{3}H]thymidine incorporation was decreased. Sample P4+AS p21 was treated with a 20-nM p21 antisense oligonucleotide (AS), which blocked the expression of p21, and sample P4+AS p27 was treated with a 20 nM p27 AS, which blocked the expression of p27. Treatment of RASMC with AS p21 or AS p27 alone did not cause any significant change in [^{3}H]thymidine incorporation into RASMCs (data not shown). Consequently, pretreatment of the RASMCs with AS p21 or AS p27 partially reversed the progesterone-induced decrease in [^{3}H]thymidine incorporation. The progesterone-induced inhibition in [^{3}H]thymidine incorporation of RASMCs was completely reversed by a combined administration of both antisense oligonucleotides to p21 and p27 together (Fig. 4).

Discussion

Progesterone has been suggested to be a paradoxical hormone existing either growth stimulatory or inhibitory effects, depending on the tissue and treatment regimen (19–23). For example, progesterone inhibits epithelial growth in the uterus (24). On the other hand, in animals with established progesterone receptor-positive mammary tumors, progesterone usually stimulates proliferation (25). We previously demonstrated that progesterone, the natural hormone, at physiologic levels inhibited DNA synthesis and decreased cell number in cultured RASMCs in a dose-dependent manner. The specificity of progesterone's inhibitory effect on [^{3}H]thymidine incorporation was confirmed by preincubation of RASMCs with progesterone receptor antagonist RU486, which antagonized the inhibition of [^{3}H]thymidine incorporation induced by progesterone (14). In the present study, we conducted [^{3}H]thymidine incorporation and flow cytometry analyses to further demonstrate that progesterone at physiologic levels (5–500 nM) inhibited DNA synthesis in cultured RASMCs in a time-dependent manner and arrested the cells at the G0/G1 phase of the cell cycle (Fig. 1).

Observation of intracellular events associated with the progression of cell cycle activity have suggested that coordinated successive activation of certain CDKs occurs late in the G1 phase and is instrumental in the transition from the G1 to the S-phase (26, 27). This CDK activation is in turn modulated by association with a series of regulatory subunits called cyclins and with a group of CDK-inhibitory proteins designated CKIs (28). Cyclins have been identified as cyclins A, D1, D3, and E, whereas the most common CDKs are designated CDK2 and CDK4. Cyclin A-CDK2 and cyclin E-CDK2 complexes form late in the G1 phase as cells prepare to synthesize DNA (29), and formation of the cyclin E complex is a rate-limiting step in the G1/S transition (30). It has been suggested that progesterone-induced entry of cells into the S-phase of the cell cycle is accompanied by transient increases of cyclin D1 and CDK4 activity (20, 21). In contrast, progesterone-induced retardation of cell entering into the S-phase is accompanied by down-regulation of cyclins D, A, and B and sequential increases in the levels of the CDK inhibitors, p21 and p27 (21). A study done in T-47D breast cancer cells demonstrated that progestin-mediated growth arrest was preceded by inhibition of cyclin D1-CDK4, cyclin D3-CDK4, and cyclin E-CDK2 kinase activities and reduced phosphorylation of pRB and p107. This was accompanied by decreases in the expression of cyclins D1, D3, and E protein; decreased abundance of cyclin D1- and cyclin D3-CDK4 complexes; increased association of the CDK inhibitor, p27, with the remaining CDK4 complexes; and changes in the molecular masses and compositions of cyclin E complexes (23).

Previously we demonstrated that progesterone at a range of concentrations (5–500 nM) dose dependently decreased the levels of cyclin A and cyclin E mRNA (14). This finding led us to propose that progesterone inhibits arterial smooth muscle cell proliferation by interrupting the cell cycle at the G1/S transition. In the present study, we further demonstrated that progesterone dose dependently decreased the levels of cyclin A, cyclin E, and CDK2 protein but not cyclin D1 and CDK4 protein. Moreover, treatment of RASMCs with progesterone resulted in an increase in the levels of p21 and p27 protein at 24 h after treatment. In accord with the established notion that p21 and p27 are two known CDK inhibitors, we found in progesterone-treated cells that the formations of the CDK2-p21 and CDK2-p27complex were increased, and the assayable CDK2 kinase activity was decreased. In contrast, the formations of the CDK4-p21 and CDK4-p27 complex and the assayable CDK4 kinase activity were not changed significantly. The progesterone effect on the CDK activity appears to be tissue specific and more analogous to the effects on the uterine epithelium than on breast cancer cells. Pretreatment of uterine epithelial cells with progesterone abrogated estrogen-induced cyclin E-CDK2 activation (31). In breast cancer cells, on the other hand, the progestin-induced

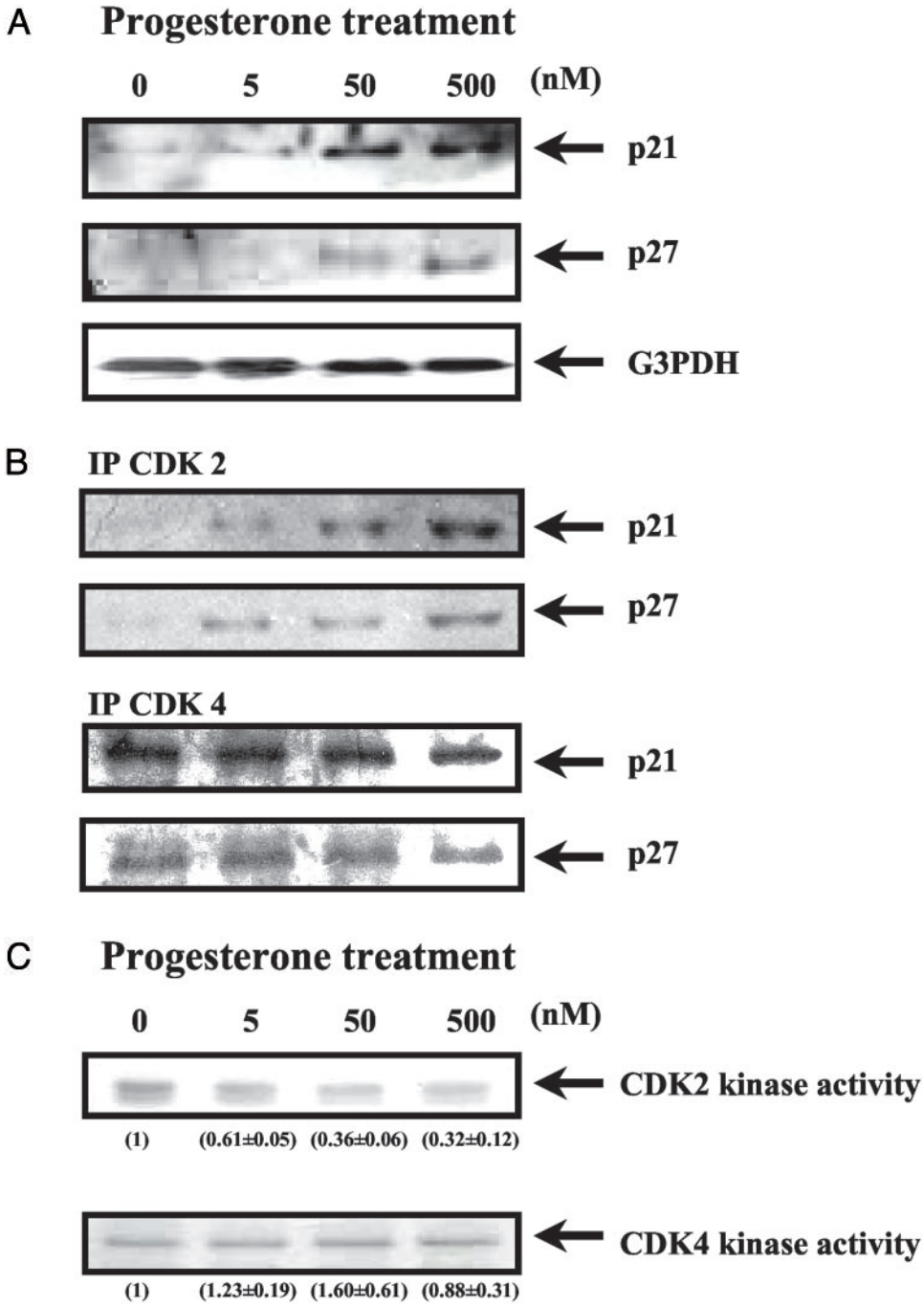

FIG. 3. Effect of progesterone on levels of CKI protein, CKI-CDK association, and CDK kinase activity. A, Progesterone dose dependently increases the levels of p21 and p27 protein in RASMCs. Membrane was probed with anti-G3PDH antibody to verify equivalent loading. B, Treatment of RASMCs with progesterone (5–500 nM) induced up-regulation of the formations of CDK2-p21 and CDK2-p27 complex in a dose-dependent manner. The formations of CDK4-p21 and CDK4-p27 complex were not affected by progesterone treatment. CDK2 was immunoprecipitated by anti-CDK2 antibody, and CDK2-p21 complex was detected by anti-p21 antibody, whereas CDK2-p27 complex was detected by anti-p27 antibody. CDK4 was immunoprecipitated by anti-CDK4 antibody, and CDK4-p21 complex was detected by anti-p21 antibody, whereas CDK4-p27 complex was detected by anti-p27 antibody. C, The CDK2 kinase activity was decreased dose dependently by progesterone treatment, whereas CDK4 kinase activity was not significantly changed. Results from a representative experiment are shown. Mean values of CDK2 and CDK4 enzyme activity from three experiments are shown in the *parentheses* (means ± SEM). The CDK2 and CDK4 kinase activity were determined as described in *Materials and Methods.*

growth inhibition was preceded by inhibition of cyclin D1-Cdk4, cyclin D3-Cdk4, and cyclin E-Cdk2 kinase activities (19, 23, 32).

Previously, it has been demonstrated that regulation of transcription of the p21 promoter is involved in the progesterone-induced cell cycle arrest at the transition of the cells from the G1 phase to the S-phase (33). The important role of p21 and p27 play on the progesterone-induced inhibition of DNA synthesis was further confirmed by the demonstration that pretreatment of the RASMCs with a p21 or p27 antisense oligonucleotide reduced the progesterone-induced inhibition of [^{3}H]thymidine incorporation into RASMCs. Moreover, a combined treatment of RASMC with p21 and p27 antisense oligonucleotides completely reversed the progesterone-induced inhibition of [^{3}H]thymidine incorporation. Accordingly, we concluded that progesterone may inhibit RASMCs proliferation by increasing the levels of p21 and p27 protein, which in turn inhibit CDK2 kinase activity, and finally impair the transition of the cells from the G1 phase to the S phase.

In conclusion, the results from the present study indicate that progesterone-induced cell cycle arrest in RASMCs occurred when the cyclin-CDK system was inhibited just as p21 and p27 protein levels were augmented. Although animal

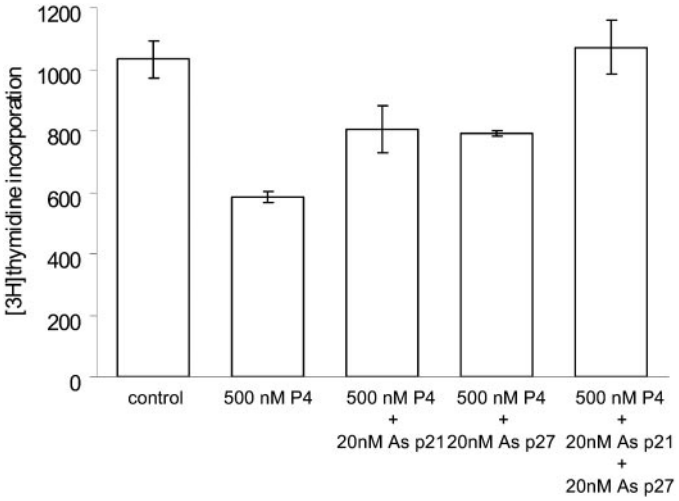

FIG. 4. Involvement of p21 and p27 in the progesterone-induced decrease of [^{3}H]thymidine incorporation. Antisense p21 or p27 oligonucleotide alone partially reversed the progesterone-mediated decrease of [^{3}H]thymidine incorporation. However, the progesterone-induced inhibition in [^{3}H]thymidine incorporation of RASMCs was completely reversed by a combined administration of both antisense oligonucleotides to p21 and p27 together. AS p21 or p27 was added to RASMCs at a final concentration up to 20 nM at 1 h before the cell was challenged with 2% FBS, and 500 nM progesterone for an additional 24 h. [^{3}H]Thymidine incorporation was conducted after RASMC release from quiescence by incubation in culture media supplemented with 2% FBS and PBS (control) or 500 nM progesterone in PBS. Three to four samples were analyzed in each group, and values represent the means ± SEM. P4, Progesterone; AS p21, antisense p21 oligonucleotide; AS p27, antisense p27 oligonucleotide.

studies of progesterone-mediated antiatherosclerosis are still ongoing, the findings from the present studies suggest the potential applications of progesterone in the treatment of atherosclerosis.

Acknowledgments

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