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Effects of lovastatin on expression of cell cycle regulatory proteins in vascular smooth muscle cells

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Background. The sequential appearance of cyclins D and E is thought to initiate subsequent DNA synthesis in proliferating cells. Previous studies have reported that DNA synthesis in cultured rat vascular smooth muscle cells (VSMCs) was suppressed by the HMG-CoA reductase inhibitor lovastatin. The effects of lovastatin on cell cycle regulatory proteins in proliferating VSMCs, however, are largely unknown. Thus, we investigated the sequential expression of cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 4, CDK2, and p27Kip1 in cultured rat VSMCs stimulated by platelet-derived growth factor (PDGF)-BB in the presence or absence of lovastatin.

Methods. Quiescent VSMCs, with and without lovastatin (20 μM) pretreatment for nine hours, were stimulated by PDGF-BB (25 ng/ml). The incorporation of tritiated thymidine was done to assess DNA synthesis. VSMC lysates were obtained every 6 hours for up to 36 hours after stimulation and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis using relevant polyclonal antibodies. Autoradiograms were analyzed using a densitometer.

Results. The peak expression of cyclins D1 and E occurred at 18 and 30 hours of PDGF stimulation, respectively. Concomitant expression of CDK4 and CDK2 was also observed. The expression of p27Kip1, by contrast, was reduced in association with DNA synthesis. Lovastatin suppressed DNA synthesis and reduced the expression of cyclin D1 and cyclin E, whereas p27Kip1 expression was strongly induced by lovastatin pretreatment. CDK4 and CDK2 expression was unaffected by lovastatin treatment.

Conclusions. PDGF-BB induces cyclins D1 and E prior to the onset of DNA synthesis in VSMCs. Lovastatin may suppress DNA synthesis in VSMCs by inducing p27Kip1 and reducing expression of cyclins D1 and E.

Intimal hyperplasia is a histologically characteristic feature in the arterial tree of chronic renal allograft rejection [1]. The development of this lesion involves the proliferation and migration of vascular smooth muscle cells (VSMCs) caused by mitogens such as platelet-derived growth factor (PDGF) [2]. Previous studies have reported that HMG-CoA reductase inhibitors diminish the prenylation of G-γ subunits and p21*ras* [3] and inhibit VSMC proliferation [4]. Cell proliferation is regulated at the level of the cell cycle by specific cyclins, cyclin-dependent kinases (CDKs), and cyclin kinase inhibitors (CKIs) [5–7]. In order to clarify the effects of HMG-CoA reductase inhibitors on the VSMC cell cycle, we investigated the effects of lovastatin, an HMG-CoA reductase inhibitor, on DNA synthesis and the expression of cyclins D and E, CDKs 2 and 4, and the CKI p27Kip1.

Key words: CDK2, CDK4, cyclin D1, cyclin E, DNA synthesis.

METHODS

Cell culture

Cultured rat VSMCs at the fifth and seventh passage in 175 cm^2 flasks were studied. Subconfluent cells were synchronized to quiescence by culture in medium containing 0.2% heat-inactivated fetal bovine serum (HIFBS) for 48 hours. Some cells were then stimulated by PDGF-BB (25 ng/ml) in 0.2% HIFBS, whereas control cells remained in 0.2% HIFBS. To study the effects of lovastatin, some VSMCs were pretreated with lovastatin (20 μM in 0.2% HIFBS) during the last nine hours of synchronization. Lovastatin did not alter cell shape or viability (viability was more than 95%).

DNA synthesis

Quiescent VSMCs were dispersed in 96-well multiplates under the same conditions described earlier. During the last six hours of incubation, 1 μCi of [^{3}H]TdR (20.0 Ci/mmol; New England Nuclear, Boston, MA, USA) was added to respective wells, and [^{3}H]TdR incorporation was measured with a liquid scintillation counter and used as an index of DNA synthesis.

Cell lysate

Vascular smooth muscle cells were rinsed three times with cold phosphate-buffered saline and lysed at 0, 6, 12, 18, 24, 30, and 36 hours of PDGF stimulation. The lysate solution contained 1% Triton X-100, 10% glycerol, 20 mM HEPES, 100 mM NaCl, 10 mg/ml leupeptin,

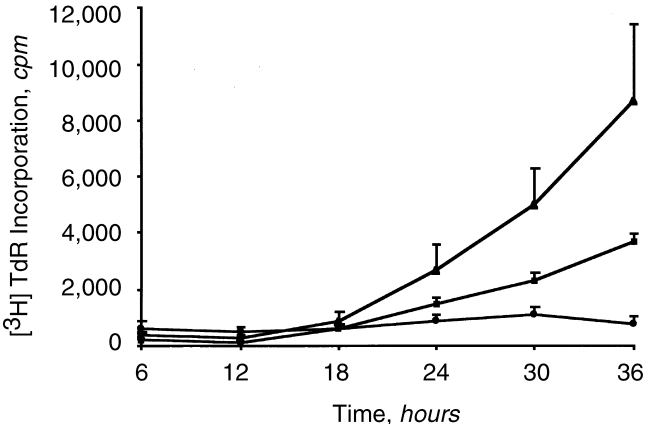

Fig. 1. DNA synthesis in cultured rat vascular smooth muscle cells (VSMCs) stimulated by platelet-derived growth factor (PDGF). DNA synthesis was suppressed by lovastatin pretreatment for nine hours prior to PDGF stimulation. Triangles, VSMCs + PDGF 25 ng/ml; squares, VSMCs + lovastatin (20 μM) pretreatment prior to PDGF stimulation; circles, control VSMCs maintained in 0.2% heat-inactivated fetal bovine serum. Data represent means ± SD of three experiments.

10 mg/ml antipain, 10 mg/ml pepstatin, 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride. After centrifugation at 12,000 r.p.m. for five minutes at 4°C, the protein concentration of the supernatant was measured using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL, USA).

Western blot analysis

Fifty μg of protein from each cell lysate was separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 0.5% casein, the membranes were hybridized with rabbit primary polyclonal antibodies to cyclins, CDKs, and p27Kip1 for 60 minutes at room temperature, followed by hybridization with secondary horseradish peroxidase-conjugated goat antirabbit antibody (1:20,000; Chemicon International, Temecula, CA, USA) for 60 minutes at room temperature. The primary antibodies used in this study were cyclin D1 (1:1500; Upstate Biotechnology, Lake Placid, NY, USA), cyclin E (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CDK4 (1:3000; Santa Cruz Biotechnology), CDK2 (1:500; Santa Cruz Biotechnology), and p27 (1:1000; Santa Cruz Biotechnology). Autoradiograms of the Western blots were analyzed using a Personal Densitometer SI and the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA, USA).

RESULTS

Thymidine incorporation in control, quiescent VSMCs remained at a low and constant level throughout the experimental period (Fig. 1). DNA synthesis in VSMCs stimulated by PDGF appeared to begin between 18 and 24 hours after exposure to PDGF and increased progressively thereafter (Fig. 1). Lovastatin suppressed DNA synthesis in PDGF-stimulated VSMCs.

Increased expression of cyclin D1 was found at 6 hours, and peak expression of cyclin D1 was observed at 18 hours after PDGF stimulation (Fig. 2A). Lovastatin suppressed cyclin D1 expression at 6 and 12 hours, whereas the peak expression of cyclin D1 at 18 hours was similar to that in cells not treated with lovastatin. Cyclin D1 expression at 36 hours in PDGF-stimulated VSMCs, regardless of whether cells were exposed to lovastatin, was similar to that in control cells (Fig. 2A). Cyclin E expression in PDGF-stimulated VSMCs increased progressively to a peak at 30 hours (Fig. 2B). Lovastatin reduced the peak cyclin E expression by more than 40%.

A time-dependent increase in CDK4 expression was found up to a peak at 24 hours of PDGF stimulation, followed by a subsequent decline (Fig. 2C). Lovastatin did not affect CDK4 expression. CDK2 expression in PDGF-stimulated VSMCs increased throughout the experimental period (Fig. 2D). At 36 hours, CDK2 expression was increased nearly threefold compared with that in control cells. Lovastatin did not suppress CDK2 expression.

Quiescent VSMCs at the end of the 48-hour synchronization period displayed high expression of the CKI p27Kip1 (Fig. 2E). Lovastatin pretreatment increased p27 expression by 100%. Exposure of VSMCs to PDGF caused a progressive decline in p27 expression throughout the experimental period.

DISCUSSION

Cyclins and CDKs constitute the major positive cell cycle regulatory proteins [6–8]. Cyclins D and E are synthesized in the G1 phase, and the latter is essential for G1 to S phase transition [9]. Cyclins D and E bind to CDK4 and CDK2, respectively, leading to the inactivation of retinoblastoma protein and progression of the cell cycle [10, 11]. Cyclin D-CDK4 complexes are inhibited by all CKIs, whereas cyclin E-CDK2 complexes are inhibited by the CKI p27Kip1, causing cells to remain at the G1 phase and quiescent [8].

In this study, DNA synthesis appeared to begin between 18 and 24 hours of exposure of VSMCs to PDGF. Prior to the onset of DNA synthesis, VSMCs displayed increased expression of cyclins D1 and E. Cyclin D1 expression was increased at 6 hours of PDGF stimulation, peaked at 18 hours of stimulation, and declined thereafter. Cyclin E expression increased steadily to a peak at 30 hours and then declined by 36 hours. The observed time course of changes in cyclins D1 and E was consistent, therefore, with the regulatory roles of these proteins in the cell cycle. Cyclin D1 is synthesized

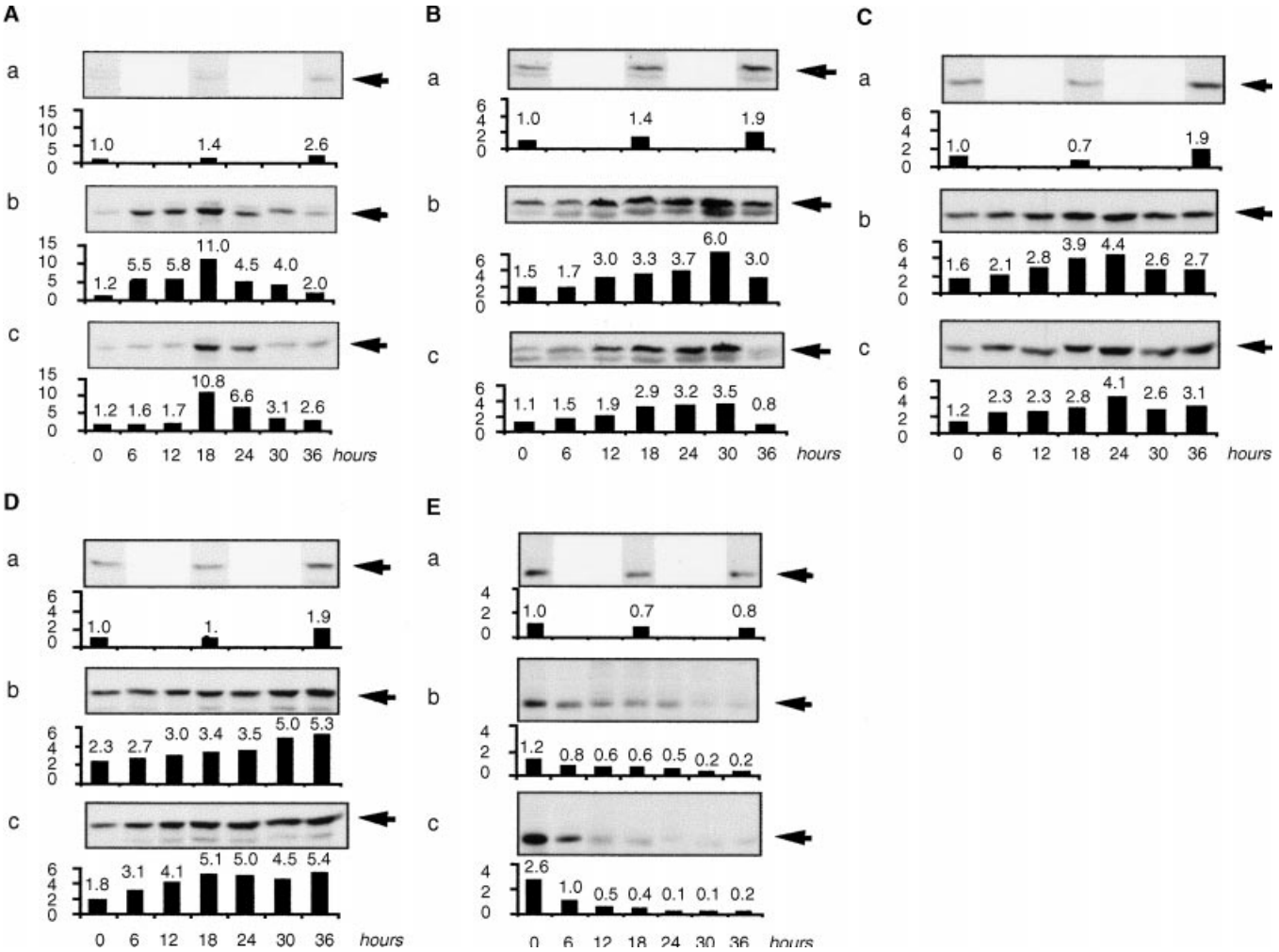

Fig. 2. Western blot analysis of expression of cell cycle regulatory proteins in cultured rat vascular smooth muscle cells (VSMCs). (*A*) Cyclin D1. (*B*) Cyclin E. (*C*) CDK 4. (*D*) CDK2. (*E*) p27Kip1. Molecular weight of each cell-cycle protein is cyclin D1, 36 kDa; cyclin E, 42 and 50 kDa; CDK4, 34 kDa; CDK2, 33 kDa. Culture conditions are (a) control VSMCs incubated in 0.2% heat-inactivated fetal bovine serum; (b) VSMCs + PDGF (25 ng/ml); and (c) VSMCs + lovastatin (20 μM) pretreatment for nine hours prior to PDGF stimulation. Optical density (O.D.) of the autoradiogram at time 0 was determined as baseline (O.D. = 1.0). Data in graphs represent the ratios of O.D. values.

when quiescent (G_0) cells are stimulated to enter the G1 phase, and cyclin E is necessary for transition from the G1 to the S phase, the phase of DNA synthesis.

Associated with increased expression of cyclins D1 and E in PDGF-stimulated VSMCs was enhanced expression of the respective CDKs to which the cyclins bind. Indeed, the time course of increased CDK4 and CDK2 expression paralleled the time course of increased cyclin D1 and cyclin E expression, respectively. PDGF stimulation of VSMCs also caused a rapid decline in expression of the CDK inhibitor p27Kip1, consistent with release of cells from G_0 and their entry into the cell cycle.

Lovastatin pretreatment of VSMCs prior to PDGF stimulation markedly enhanced the expression of p27Kip1, reduced the early (6 to 12 hr) expression of cyclin D1, and reduced cyclin E expression. One or more of these actions of lovastatin may have been responsible for the effect of lovastatin to suppress DNA synthesis in VSMCs exposed to PDGF. By contrast, lovastatin suppression of DNA synthesis did not appear to depend on a reduction in CDK4 or CDK2 expression.

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