Distinct Role of cAMP and cGMP in the Cell Cycle Control of Vascular Smooth Muscle Cells

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Abstract—cAMP and cGMP are known to suppress vascular smooth muscle cell (SMC) proliferation. In this study, our aim was to delineate the molecular mechanism underlying cAMP and cGMP suppression of cell cycle transition in human SMCs. cAMP inhibits both platelet-derived growth factor–stimulated cyclin-dependent kinase (cdk) 2 and cdk4 activation through upregulation of the cdk2 inhibitor p27Kip1 and downregulation of cyclin D1 expression, which leads to a complete arrest of the cells in phase G1. In contrast, cGMP inhibits cyclin D1 expression, inhibits cdk4 activation, and delays platelet-derived growth factor–mediated cdk2 activation, resulting in a delay in G1/S transition. A transient increase in p27Kip1 in cdk2 immunoprecipitates, without changes in total cellular p27Kip1 levels, correlates with the delay in cdk2 activation caused by cGMP. Thus, cAMP and cGMP differentially affect cell cycle through distinct regulation of cell cycle molecules in human SMCs. (Circ Res. 1999;85:985-991.)

Key Words: p27Kip1 • platelet-derived growth factor • kinase

Vascular smooth muscle cells (SMCs) in the normal arterial media are quiescent, contractile, and resistant to growth factor stimuli. However, injury to the arterial wall results in a phenotypic change in the SMCs that enables them to respond to growth factors and to proliferate. The change in SMC phenotype that leads to proliferation is a key event in the progression of atherosclerotic lesions and in restenosis after angioplasty. The isolation and culture of SMCs are associated with a similar phenotypic change from a contractile to a synthetic, proliferative phenotype. The molecular mechanisms of modulation from the quiescent to the proliferative phenotype are not fully understood.

Cyclic nucleotides (cAMP and cGMP) are known to inhibit SMC proliferation. The proliferation of SMCs is regulated by several intracellular signaling pathways, and cAMP is known to inhibit many of these pathways, including mitogen-activated protein kinase cascade, p70 S6 kinase, and cyclin-dependent kinase (cdk) 4, which can ultimately inhibit cell cycle progression. Little is known about the cGMP-mediated signaling system in the regulation of SMC proliferation. However, agents that increase intracellular cGMP can attenuate mitogenesis induced by growth factors.

Cell proliferation is regulated at the level of the cell cycle by cell cycle–regulatory proteins. Under the appropriate stimulation, quiescent cells travel through the cell cycle as a consequence of the activation of specific cdks. Two families of cdk inhibitors regulate cyclin/CDK complexes. Cyclin D1/cdk4 and cyclin E/cdk2 are known to be required for G1/S transition and DNA synthesis. In this study, we compared the suppressive effect of cAMP and cGMP on SMC proliferation stimulated by platelet-derived growth factor (PDGF)-BB. Our goal was to delineate the molecular mechanisms underlying the cAMP- and cGMP-mediated suppression of SMC proliferation.

Materials and Methods

Materials
All reagents, unless otherwise stated, were purchased from Sigma Chemical Co. Recombinant PDGF-BB was purchased from Genzyme. Antibodies against cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) were obtained from Calbiochem Corp. cdk2, cdk4, and cyclin E were purchased from Santa Cruz Biochemicals. Antibodies to cyclin D1 were from Upstate Biotechnology Inc. Antibodies to p27Kip1 and p21Cip1/Waf1 were obtained from Pierce Chemical and Santa Cruz Biochemicals.

Cells and Cell Culture
Human SMCs (neonatal umbilical artery origin), obtained from Cell System Co, were cultured in Dulbecco’s modified Eagle’s medium containing 10% FCS (Life Technologies, Inc) and used for experiments within 3 to 10 passages. Before mitogenic stimulation, subconfluent cells were arrested in the quiescent state with a culture
in Dulbecco’s modified Eagle’s medium containing 0.2% FCS for 72 hours. 8-Bromo-cAMP (8-Br-cAMP) and 8-bromo-cGMP (8-Br-cGMP) were simultaneously added with mitogenic stimulation.

**Cell Cycle Transition**

Cell cycle transition was determined through bromodeoxyuridine (BrdU) or H-thymidine incorporation. BrdU (10 μmol/L) was added to SMCs just before PDGF stimulation, and the cells were cultured for an indicated number of hours. The cells were fixed in 70% ethanol, and incorporated BrdU was detected on the basis of fluorescein isothiocyanate-labeled anti-BrdU antibody. The total number of nuclei was determined on the basis of staining with propidium iodide, and the cells that had entered S phase were calculated as the percentage of BrdU-incorporated cells. H-Thymidine incorporation was determined as described previously.

**Northern Blot Analysis**

Northern blot analysis for cell cycle molecules was performed as described previously. cDNAs for mouse cyclin D1, cdk2, and cdk4 were kindly provided by Dr H. Matsushime (Tokyo University, Tokyo, Japan). Rat GAPDH cDNA was a generous gift of Dr P. Fort (Université des Sciences et Techniques du Languedoc, Montpellier Cedex, France).

**Immunoprecipitation, Western Blot Analysis, and Measurements of cdk2 or cdk4 Activity**

Cell lysate preparation and Western blot analyses were performed as previously described. Cyclin/cdk complexes were determined through immunoprecipitation with antibodies against cdk4 and cdk2, followed by immunoblotting with specific antibodies as described previously. Both cdk2 and cdk4 activities were determined with immunoprecipitation and in vitro kinase assay as previously described. Cell lysates were pre cleared with immobilized protein G-Sepharose, and cdk2 or cdk4 was immunoprecipitated with anti-cdk2 or anti-cdk4 monoclonal antibody (100 μL/sample), respectively. Histone H1 (Boehringer Mannheim) was used for a substrate for cdk2, and retinoblastoma protein (Santa Cruz Bioc chemicals) was used for a substrate for cdk4.

**Statistical Analysis**

Statistical analysis was performed with ANOVA with multiple comparisons (Scheffe test) with the use of StatView IV software.

**Results**

**Inhibitory Effect of cAMP and cGMP on Cell Cycle Transition in SMCs**

We first compared the effect of two cyclic nucleotides, 8-Br-cAMP and 8-Br-cGMP, on PDGF-stimulated cell cycle transition in human SMCs. G1/S transition in the cell cycle was determined with BrdU incorporation, in which BrdU was added together with PDGF stimulation. G1/S transition in cell cycle was determined by BrdU incorporation, in which BrdU was added together with PDGF stimulation. Each column represents mean±SD values. *P<0.05 vs 0 mmol/L (multiple comparison, Scheffe test). This experiment was performed twice, and representative data are shown.

**cGMP Preferably Inhibits cdk4 Activities, and cAMP Inhibits Both cdk4 and cdk2 Activities**

To understand the molecular basis underlying cAMP and cGMP suppression of SMC proliferation, we examined the effect of the cyclic nucleotides on cdk4 and cdk2 activities, the activation of which is shown to be essential for the G1/S transition in human SMCs. G1/S transition in cell cycle was determined by BrdU incorporation, in which BrdU was added together with PDGF stimulation. G1/S transition in cell cycle was determined by BrdU incorporation, in which BrdU was added together with PDGF stimulation. Each column represents mean±SD values. *P<0.05 vs PDGF alone (multiple comparison, Scheffe test). This experiment was performed twice, and representative data are shown.
transition. The kinetics of cdk2 and cdk4 activation after PDGF stimulation revealed that cdk2 was activated later than 18 to 24 hours, whereas cdk4 activation reached a maximal level at 18 hours (Figure 5A). The activation of cdk4 was preceded by an increase in cyclin D1 mRNA and protein levels. After PDGF stimulation, cyclin D1 mRNA was induced as early as 3 hours and reached a maximal level at 6 hours, whereas cyclin D1 protein levels were gradually increased up until 18 hours (Figure 5B).

Both 8-Br-cAMP and 8-Br-cGMP (Figure 6) equally and entirely suppressed PDGF-stimulated cdk4 activation at 18 hours. cdk4 protein levels were also increased by PDGF, and both cyclic nucleotides significantly suppressed its induction. Protein levels of cyclin D1 were induced by PDGF, and these were suppressed to basal levels by both 8-Br-cAMP and 8-Br-cGMP.

![Figure 3. Additive suppressive effect of cGMP and cAMP on SMC DNA synthesis. Quiescent SMCs were treated with PDGF (10 ng/mL) alone, PDGF plus 8-Br-cAMP, PDGF plus 8-Br-cGMP, or PDGF plus cAMP plus cGMP for 24 hours. SMC DNA synthesis was determined with 3H-thymidine incorporation assay. Each plot represents mean ± SD values. This experiment was performed twice and was reproducible.](image1)

![Figure 4. Expression of PKA and PKG in human SMCs, which were cultured until confluency and serum deprived for 2 days. Expression of PKA and PKG was determined by Western blot analysis. Lane 1 represents PKA; lane 2, PKG. This experiment was repeated with SMCs with different passages (6 and 9), and the two kinases were similarly detected.](image2)

![Figure 5. Kinetics of cdk2 and cdk4 activation and cyclin D1 expression after PDGF stimulation. Quiescent SMCs were treated with PDGF (10 ng/mL), and cdk2 activity (A), cdk4 activity (A), and cyclin D1 mRNA or protein levels (B) were determined as described in Materials and Methods. This experiment was repeated, and identical results were obtained.](image3)

![Figure 6. Suppression of cdk4 activities by both cAMP and cGMP. Quiescent SMCs were treated with PDGF (10 ng/mL), PDGF plus 8-Br-cAMP (1 mmol/L), or PDGF plus 8-Br-cGMP (1 mmol/L) for 18 hours. cdk4 activity, cdk4 protein, cyclin D1 protein, and cyclin D1 mRNA levels were determined as described in Materials and Methods. B, Summary of results from 3 independent experiments. Each column represents mean ± SD values. *P < 0.05 vs control; **P < 0.05 vs PDGF (multiple comparison, Scheffé test).](image4)
8-Br-cGMP (Figure 6). The suppression of cyclin D1 protein expression by cyclic nucleotides was associated with significantly decreased levels of cyclin D1 mRNA, suggesting that both cyclic nucleotides inhibit cyclin D1 expression at an mRNA level.

We next examined the effect of cyclic nucleotides on PDGF-stimulated cdk2 activation. cAMP completely suppressed cdk2 activation at 18 and 24 hours (Figure 7). cGMP suppressed PDGF-induced cdk2 activation at 18 hours, but its inhibitory effect was not continued until 24 hours (Figure 7). To explore the possibility that cGMP delays cdk2 activation, the kinetics of PDGF-stimulated cdk2 activation were examined in the presence of vehicle, cAMP, or cGMP. As shown in Figure 8, cGMP delayed, but did not block, the activation of cdk2 induced by PDGF. In contrast, cAMP significantly inhibited the PDGF-stimulated cdk2 activation at all time points. At 18 hours, the protein levels of cdk2 were increased 50% by PDGF, but its level was not suppressed by either cAMP or cGMP (Figure 7). The faster migrating form of cdk2 on Western blotting was phosphorylated on Thr160, which has been shown to be essential for cdk2 activation.24 The density of phosphorylated cdk2 was induced by PDGF at 18 hours, and this induction was significantly but partially suppressed by either cAMP or cGMP (Figure 7). Neither cAMP nor cGMP affected the protein levels of cyclin E, which forms a complex with cdk2 and contributes to cdk2 activation in the late G1 phase.13

cAMP, but Not cGMP, Inhibits PDGF-Mediated Downregulation of p27Kip1

To investigate how cAMP and cGMP differentially regulate cdk activation and cell cycle transition, we examined regulation of the candidates for cdk inhibition: p21Cip1/Waf1 and p27Kip1.12 We examined the levels of cdk inhibitors complexed with cdk4 or cdk2 at 18 hours, when cdk2 and cdk4 activities were significantly suppressed by both cAMP and cGMP. Western blot analysis showed that PDGF treatment for 18 hours downregulated p27Kip1 protein levels and that cAMP prevented this p27Kip1 suppression (Figure 9). Consistent with the high level of total cellular p27Kip1, p27Kip1 levels associated with cdk4 and cdk2 were significantly higher in
the presence of cAMP (Figure 9). The effect of cAMP on total cellular and cdk2-bound p27Kip1 levels was consistent up to 30 hours (Figure 9).

In contrast to cAMP, cGMP did not have a significant effect on total cellular p27Kip1. Instead of increasing, it decreased cdk4-associated p27Kip1 levels at 18 hours. However, p27Kip1 levels associated with cdk2 in cGMP-treated cells were higher than those in vehicle-treated cells, suggesting a dynamic shift in p27Kip1 from cdk4 to cdk2 (Figure 9). The cGMP-induced increase in cdk2-bound p27Kip1 levels was transient and was not observed at 30 hours (Figure 9).

Discussion

We examined the molecular basis underlying cAMP and cGMP regulation of SMC proliferation and clearly showed that the main effect of cGMP is suppression of cyclin D1 and cdk4 activity, which leads to a delay in cdk2 activation and G1/S transition in the cell cycle. In contrast, cAMP inhibits both cdk4 and cdk2 activities, leading to complete cell cycle arrest in G1.

It is well documented that cGMP-generating reagents have an antimitotic effect on cell cycle progression in vitro and in vivo.9,10 Because cAMP is also known to strongly suppress cell cycle transition in vascular SMCs,25–27 we sought to examine and delineate the molecular basis for cAMP and cGMP suppression of SMC proliferation. Interestingly, SMCs treated with cGMP do not enter the S phase 24 hours after PDGF stimulation, whereas at 30 hours, a significant portion of the cells travel to the S phase. This result suggests that cGMP delays, but does not block, the G1/S transition, probably through the prolongation of G1. In contrast, cAMP appears to arrest the cells in G1 as previously described,8 because the percentage of cells that have entered the S phase after PDGF stimulation is proportionally suppressed at each time examined. It has been reported that PKG is downregulated in SMCs in culture.21 It is unlikely that the distinct effect of cAMP and cGMP is due to the downregulation of PKG, because both PKG and PKA are abundantly expressed in our human SMC system. Because even the lower concentrations of cAMP and cGMP have additive suppressive effects on SMC proliferation (Figure 3), several antimitogenic agents, including vasodilators, may have a cooperative role in the regulation of SMC proliferation with the use of distinct PKA and PKG systems. These findings led us to examine possible distinct regulation of cell cycle molecules by cAMP and cGMP.

It has been proposed that cdk activities control the G1/S transition in mammalian cells.13 Both D- and E-type cyclins are known to be important regulators in G1/S control, even though some reports raise the possibility that they have distinct roles.17,28 In many cell systems, the induction of cyclin D after growth factor stimulation precedes that of cyclin E. Thus, cyclin D–associated kinase is maximally activated earlier than cyclin E–associated kinase.13 cdk4 is a main catalytic partner of cyclin D, and cdk2 is known to form complexes with cyclin E and cyclin A. In the present study in human SMCs, cdk4 activation after PDGF stimulation occurs as early as 12 hours and reaches a maximal level at 18 hours, and cdk2 activities follow those of cdk4. Thus, the distinct kinetics of the activation of these cskks support the idea that they have different roles in cell cycle regulation. Cyclin D1 expression precedes the activation of cdk4, and cdk4 protein levels are also significantly increased after PDGF stimulation. Although cdk2 activities are dramatically induced after treatment with PDGF in human SMCs, cyclin E protein levels do not oscillate. These results in human SMCs confirm previous findings in various SMC systems concerning the growth factor regulation of cell cycle molecules.19,21,29,30

To explore the possibility that cdk4 or cdk2 activities contribute to the distinct effects of cAMP and cGMP on cell cycle transition, we examined the effects of the cyclin nucleotides on cdk4 and cdk2 activities. cAMP is known to inhibit cdk4 activity in macrophages,8 and in SMCs, cyclin D1 expression is known to be partially inhibited by cAMP.27 There was only one report of cGMP regulation of the cell cycle in rat SMCs, in which cyclin A and cyclin D expressions are suppressed by cGMP.30 No reports, however, have compared the detail regulation of cell cycle molecules underlying cAMP and cGMP suppression of SMC proliferation. In the human SMC system, both cAMP and cGMP entirely suppress PDGF-stimulated cdk4 activity at 18 hours, which is associated with the suppression of cyclin D1 mRNA, cyclin D1 protein, and cdk4 protein levels. PDGF-stimulated cdk2 activation is completely inhibited by cAMP, whereas the kinetics of cdk2 activation are delayed by cGMP. Thus, altered kinetics in cdk2 activation may account for delayed G1/S transition in cells treated with cGMP. Even though PDGF-stimulated cdk2 activities at 18 hours are almost entirely suppressed by cGMP or cAMP, the levels of cyclin E are not dramatically regulated by cyclic nucleotides. However, cAMP and cGMP significantly suppress cdk2 phosphorylation induced by PDGF. These data imply the involvement of cdk2 inhibitors in the inactivation of the cyclin E/cdk2 complex.31

A family of cdk inhibitors plays a major role in the cell cycle machinery.12,32,33 Two molecules, p21Cip1/Waf1 and p27Kip1, directly inhibit cdk activity and prevent its phosphorylation. p27Kip1 has been shown to mediate cell cycle arrest in response to various factors, including transforming growth factor-β,34 rapamycin,35 cAMP,8 and extracellular matrices.21 On the other hand, p21Cip1/Waf1 appears to be involved in cell cycle arrest induced by irradiation or UV irradiation.36,37 p21Cip1/Waf1 is also known to regulate senescence, apoptosis, or differentiation of various cells.38–41 The expression of p27Kip1 and p21Cip1/Waf1 in vascular injury was extensively studied and showed the possible roles of the inhibitors in SMC behavior in SMCs in vivo.42–44 Moreover, exogenous overexpression of the inhibitors in vessel wall successfully inhibits injury-induced SMC proliferation.53,46

In this study, p21Cip1/Waf1 is barely detected and regulated in SMCs. p27Kip1 is abundantly expressed in serum-starved cells, and its level is downregulated by PDGF as previously described.29,47 cAMP successfully interferes with the PDGF-directed decrease in p27Kip1 in total cellular lysates or cdk4 immunoprecipitates, which is in good agreement with previ-
ous observations. Because p\textsuperscript{27}Kip\textsubscript{1} complexed with cdk2 or cdk4 is higher in cAMP-treated cells than in vehicle-treated cells, p\textsuperscript{27}Kip\textsubscript{1} levels appear to account for the entire suppression of both cdk activities, which leads to G\textsubscript{i} arrest in the cell cycle. In contrast, cGMP fails to prevent PDGFinduced decrease in total cellular p\textsuperscript{27}Kip\textsubscript{1} levels; in addition, instead of increasing, it decreases the level of p\textsuperscript{27}Kip\textsubscript{1} in cdk4 immunoprecipitates. However, p\textsuperscript{27}Kip\textsubscript{1} associated with cdk2 in cGMP-treated cells is significantly higher than that in vehicle-treated cells at 18 hours, but this effect of cGMP is not maintained until 30 hours. Thus, this transient shift in p\textsuperscript{27}Kip\textsubscript{1} to cdk2 may explain how cGMP delays the activation of cdk2. It is well documented that p\textsuperscript{27}Kip\textsubscript{1} is mainly pooled in cyclin D1 complex in growing cells. Within proliferation-inhibitory stimuli such as lovastatin treatment, cyclin D1 is degraded and p\textsuperscript{27}Kip\textsubscript{1} is rapidly redistributed to cdk2, leading to the suppression of cdk2 activation and the inhibition of the cell cycle transition.\textsuperscript{48} cGMP suppression of cyclin D1 levels could transiently redistribute p\textsuperscript{27}Kip\textsubscript{1} to cdk2, resulting in a delay in cdk2 activation and G\textsubscript{i}/S transition. In the present study, it was not clear why the shift of p\textsuperscript{27}Kip\textsubscript{1} to cdk2 did not completely arrest the cell cycle. However, it may be possible that upregulation of total cellular p\textsuperscript{27}Kip\textsubscript{1} is necessary for the permanent suppression of cdk2 activity and G\textsubscript{i}/S transition. Taken together, p\textsuperscript{27}Kip\textsubscript{1} upregulation appears to be a main target for cAMP-mediated signaling, whereas downregulation of cyclin D1 and cdk4 activities could account for a delay in cell cycle transition in cGMP-treated cells.

Recently, NO donors sodium nitroprusside and \textit{S-nitroso-\textit{N}-acetylpenicillamine} have been shown to block cdk2 activation without affecting protein levels of cdk2 or cyclin E.\textsuperscript{11,49} Guo et al\textsuperscript{11} showed that the suppressive effects of NO donors on cdk2 activities are sustained until 48 hours, and Iishida et al\textsuperscript{49} suggested that p\textsuperscript{21}\textsuperscript{Sp1/Waf} can be a target of NO. It is known that the effect of NO is transduced through cGMP.\textsuperscript{50} Yu et al\textsuperscript{49} and Kronemann et al\textsuperscript{50} showed in rat SMCs that cGMP completely suppressed epidermal growth factor– or FCS-induced DNA synthesis, respectively. In our human SMC system, 1 mmol/L cGMP delayed, but did not block, PDGF-stimulated cdk2 activation and DNA synthesis. Moreover, p\textsuperscript{21}\textsuperscript{Sp1/Waf} is barely detected in our SMC system. The lack of data from the present study showing complete inhibition of SMC proliferation may be due to differences in species or SMC phenotype. It may also be possible that expression levels of PKG differ in rat SMCs. Potential unidentified NO-mediated signaling other than cGMP could also contribute to differences in effect between NO donors and cGMP.

In summary, cGMP and cAMP differentially affect cell cycle through distinct regulation of cell cycle molecules in human SMCs. A main target of cGMP appears to be the suppression of cyclin D1 expression, which leads to cdk4 inactivation and a delay in cdk2 activation and G\textsubscript{i}/S transition in the cell cycle.

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