Cyclic AMP Inhibited Proliferation of Human Aortic Vascular Smooth Muscle Cells, Accompanied by Induction of p53 and p21

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Abstract—Although cAMP is an important second messenger that plays a pivotal role in the regulation of platelet aggregation and dilatation of blood vessels, little is known about the action of cAMP on the growth of vascular smooth muscle cells (VSMCs). Thus, we initially studied the effects of cAMP accumulation by using various cAMP stimulants, including a phosphodiesterase type 3 inhibitor (cilostazol) on human aortic VSMC growth. Accumulation of cAMP inhibited the platelet-derived growth factor (PDGF)-stimulated VSMC growth in a dose-dependent manner (P<0.01), whereas PDGF significantly stimulated the growth of human VSMCs. Thus, we focused on the role of cell cycle regulatory genes, especially on a negative regulator, an anti-oncogene, p53. The protein of p53 was potentiated by cilostazol as well as forskolin and 8-bromo-cAMP, whereas PDGF decreased p53 expression. Upregulation of p53 protein by cAMP was further confirmed by the observation that the decrease in p21, a p53-inducible protein, by PDGF was significantly attenuated by cilostazol in a dose-dependent manner (P<0.01). These results revealed that accumulation of cAMP inhibited VSMC proliferation, which was at least in part due to an increase in p53-p21 expression. Because p53 and p21 have been reported to induce apoptosis, we examined apoptotic cells for cAMP accumulation. Incubation of VSMCs with cilostazol resulted in a significant increase in apoptotic cells in a dose-dependent manner compared with vehicle treatment as assessed by nuclear chromatic morphology (P<0.01); forskolin also stimulated apoptotic cells. Consistent with nuclear staining, DNA fragmentation in VSMCs treated with forskolin as well as 8-bromo-cAMP and cilostazol was significantly increased compared with DNA fragmentation in VSMCs treated with vehicle, whereas PDGF significantly decreased the rate of DNA fragmentation (P<0.01). Overall, these results demonstrated that cAMP inhibited the proliferation of human aortic VSMCs, accompanied by p53-p21-mediated apoptosis. Analogues of cAMP that have direct inhibitory effects on VSMC proliferation can be considered as potential antiproliferative drugs against VSMC growth. (Hypertension. 2000;35[part 2]:237-243.)

Key Words: atherosclerosis • restenosis • remodeling • apoptosis • cyclic AMP

Cyclic AMP is an important second messenger that plays a pivotal role in the regulation of glucose metabolism, platelet aggregation, and dilatation of blood vessels. cAMP is synthesized from ATP by adenylate cyclase and converted to 5'-AMP by phosphodiesterase (PDE). Therefore, one of the rate-limiting steps in the cascade of the cAMP pathway is PDE. Recent studies have shown the presence of 7 different subtypes in the PDE family, especially PDE type 3 (known as cAMP-PDE), which degrades cAMP and is inhibited by cGMP. PDE type 3 mainly exists in platelets, vascular smooth muscle cells (VSMCs), cardiac myocytes, and adipose tissue. Therefore, PDE type 3 inhibitors (PDE inhibitors) are well known to inhibit platelet aggregation and induce vasorelaxation through activation of cAMP. Cilostazol has been developed as a PDE inhibitor that is very potent, and it is currently used in clinical practice to treat patients with peripheral vascular disease. In contrast, prostaglandins I₂ and E are also used to treat patients with atherosclerosis through stimulation of cAMP. These agents are widely used to treat patients with peripheral vascular disease. In addition to VSMC relaxation, inhibition of VSMC proliferation by these agents has been reported. More recently, Indolfi et al. have reported that activation of cAMP–cAMP-dependent protein kinase (PKA) signaling inhibits neointimal formation after vascular injury in vivo as a model of restenosis after angioplasty. Indeed, recent clinical studies have demonstrated the clinical usefulness of cilostazol as a cAMP analogue to treat human restenosis. Therefore, it is important to note how cAMP inhibits VSMC growth. However, the molecular mechanisms of inhibition of VSMC growth by cAMP accumulation have not yet been clarified. Therefore, we examined the inhibitory mechanisms of cAMP on VSMC growth in the present study.
Methods

Cell Culture

Human aortic VSMCs (passage 5) were obtained from Clonetics Corp and cultured in modified MCDB131 medium supplemented with 5% fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 10 ng/mL epidermal growth factor, 2 ng/mL basic fibroblast growth factor, and 1 μmol/L dexamethasone in the standard fashion. These cells showed the specific characteristics of VSMCs by immunohistochemical examination and morphological observation. All the cells were used within passages 5 and 6.

Counting of Cell Number

Human aortic VSMCs were seeded onto uncoated tissue culture plates (Corning). In the preparation of experiments for determination of cell count, the cells were grown to subconfluence. After cells reached 80% confluence, the medium was changed to fresh defined serum-free (DSF) medium containing platelet-derived growth factor (PDGF, 10 ng/mL, Biosource) or vehicle. DSF medium was supplemented with insulin (5×10⁻⁷ mol/L), transferrin (5 mg/mL), and ascorbate (0.2 mmol/L), as previously described. On day 4, an index of cell proliferation was determined by using a water-soluble tetrazolium cell counting kit (WST, Wako), because this compound produces a highly water-soluble formazan dye, which makes the assay procedure easier to perform.

Flow Cytometry

For the detection of proliferating cell nuclear antigen (PCNA) expression by flow cytometry, cells were first fixed at −10°C for 5 minutes in paraformaldehyde-lysine-periodate fixation solution. After removal from the fixation solution, cells were washed in PBS and incubated with PCNA monoclonal antibody for 30 minutes at 4°C, washed in PBS, and incubated with fluorescent isothiocyanate–conjugated rabbit anti-mouse IgG monoclonal antibody (DAKo) for 20 minutes at 4°C. An irrelevant IgG1 monoclonal antibody was used in parallel as an isotopic control (Oncogene Science) before incubation with the conjugated secondary antibody. Flow cytometric analysis was carried out on a FACScan flow cytometer. The assessment of cellular DNA content was also made with a flow cytometer. The cell cycle distribution data were obtained by using the Rectangle-Fit (R-FIT) mathematical algorithm of the FACScan/Cellfit software program in the standard manner.

Counting of Apoptotic Cells

To assay cell death by apoptosis, we used a fluorescent DNA-binding dye to define nuclear chromatin morphological features as a quantitative index of apoptosis within the cell culture system. Cells are considered apoptotic when stained with Hoechst 33342 and viewed under fluorescence microscopy. Use of a membrane-permeable (Hoechst 33342) dye in the assay allowed the determination of cell viability and plasma membrane integrity and detection of any nonapoptotic toxic or necrotic death induced in the study groups. Cells were seeded onto 6-well dishes (Laboratory-Tek) and cultured in DSF medium for 2 days to make them quiescent before subconfluence. To stain the cells for DNA, cells were incubated with Hoechst 33342 (5 μg/mL in PBS) for 20 minutes at 37°C.

Although chromatin undergoes condensation during mitosis, these cells can be readily distinguished from apoptotic cells by their uniform and equatorial pattern of chromatin condensation compared with the randomly coalesced pattern typical of apoptotic cells. To quantify apoptosis, 400 nuclei from random microscopic fields were analyzed by an observer blinded to the treatment groups. The total number of apoptotic cells in each section was summed and expressed as the percentage of total cell number. At least 10 individual sections were evaluated per slide. Samples were coded so that the analysis was performed without knowledge of which treatment the individual cells had received. These observers were blinded to other data concerning the cells, as well as to the results of the other observer. The reproducibility of the results has been reported previously. Briefly, intraobserver variability was 2.4±0.3%, and interobserver variability was 3.4±0.5% (mean±SD). Photographs were obtained with a fluorescence microscope (×200 and ×400, Olympus).

Also, we measured cellular DNA fragmentation by use of a cellular DNA fragmentation ELISA kit (Boehringer-Mannheim) to quantify apoptosis. Cultured VSMCs were incubated with 10 μmol/L bromo-deoxyuridine after 2 days of incubation with DSF medium overnight at 37°C in 5% CO₂. At 2 and 4 days after cilostazol treatment, lysing solution containing BSA, EDTA, and Tween 20 was added to each well. DNA fragments in 100 μL cell lysate supernatant were tested by ELISA. The supernatant was transferred to an anti-DNA–precotted microtiter plate and incubated for 60 minutes at 37°C. After they were washed, the samples were denatured and fixed by microwave irradiation for 5 minutes. After the microtiter plate was cooled for 10 minutes at −20°C, anti-bromodeoxyuridine peroxidase–conjugated solution was added, and the plate was incubated for 60 minutes at 37°C. Wells were again washed, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added, and the plate was incubated for 30 minutes at room temperature. Stopping solution (25 μL of 1 mol/L H₂SO₄) was then added to each well. Absorbance was measured at 450 nm (reference wavelength with 690 nm). We confirmed that an increase in apoptotic cells is associated with increased absorbance. Briefly, 10 000 apoptotic cells per well reflects absorbance of 1.5 according to the manufacturer’s recommended conditions. The sensitivity of the DNA fragmentation ELISA assay correlates well with the results of the conventional [³H]thymidine-based DNA fragmentation assay. In our experimental conditions, an increase in absorbance of 0.2 reflects an increase in cell number of 2000 apoptotic cells per well.

Western Blotting

Western blotting was performed for analysis of p53 and p21 proteins. VSMCs were seeded onto 10-cm plates (Corning). Studies of VSMC growth were conducted with confluent cells made quiescent. Then, after incubation in DSF medium or PDGF (2.5 ng/mL) for 48 hours with or without cAMP-inducing agents, the cells were fixed with 10% trichloroacetic acid in saline, followed by extraction of total protein with lysis buffer (9 mol/L urea, 2% Triton X, and 5% 2-mercaptoethanol). Samples containing 100 μg protein were run on 12.5% SDS-polyacrylamide gels. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane (Hybond ECL, Amersham), and incubated with a monoclonal antibody to p53 (1:2,000, Calbiochem) or p21 (10 μg/mL, Oncogene Science) at 4°C overnight. Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham) and Hyperfilm-MP (Amersham). To quantify and compare levels of proteins, we measured the density of each band by densitometry (Shimazu). Amounts of loaded proteins were equal, as confirmed by staining with Coomassie brilliant blue R (Sigma Chemical Co). Staining with Coomassie brilliant blue R revealed identical amounts of protein in all samples for Western blotting (data not shown). Western blotting of tubulin with anti-tubulin antibody (anti-human IgG, 1:100, Oncogene) was also performed to confirm that amounts of loaded protein were equal.

Measurement of Intracellular cAMP Level

VSMCs were grown to confluence in DSF medium with 0.5% fetal calf serum. Cells were then exposed to PDGF with or without cAMP-inducing agents for 48 hours. Intracellular cAMP was measured by use of an enzyme immunoassay kit from Amersham. In brief, culture medium was removed at the indicated times, and cells were washed twice in PBS and a third time in the same buffer containing 3-isobutyl-1-methylxanthine. Cells were then lysed by the addition of ice-cold trichloroacetic acid (5%). The trichloroacetic acid–soluble supernatant was removed from the well, extracted 3 times with 10 mL ether, dried (SpeedVac), and resuspended in 0.4 mL per sample of sodium acetate buffer (pH 6.2). The enzyme immunoassay was then performed.

Materials

Cilostazol was donated by Otsuka Pharmaceutical Co (Osaka, Japan).
cilostazol, a well-known cAMP stimulant used for the treatment of
VSMCs, was examined in more detail by use of PDGF. Recently, cell
cycle regulation has been shown to be important in the growth of
VSMCs, in view of the fact that PDGF. Initially, we examined the
effect of cAMP analogues on the growth induced by PDGF in a
dose-dependent manner (Figure 1). Forskolin (30 \text{ng/mL}) as well
as 8-bromo-cAMP, PDGF (2.5 \text{ng/mL})–stimulated VSMCs
with cilostazol; PDE3 inhibitor (+), PDGF-stimulated VSMCs
treated with cilostazol (10^{-6} \text{ mol/L}); and OD, optical density.

**P<0.01 vs Untreat; #P<0.05 and ##P<0.01 vs PDGF. Cell number
of untreated VSMCs as control is 333 800±10 320. b, Inhibitory
effect of cilostazol on VSMC (n=8 per group) growth stimulated
by PDGF at 2.5 and 10 ng/mL. PDE3 inhibitor (−) indicates
PDGF-stimulated VSMCs without cilostazol; PDE3 inhibitor (+),
PDGF-stimulated VSMCs treated with cilostazol (10^{-7} \text{ mol/L});
and OD, optical density. **P<0.01 vs PDGF (0 ng/mL); #P<0.05
and ##P<0.01 vs PDE3 inhibitor (−).

Statistical Analysis
All values are expressed as mean±SEM. ANOVA with a subsequent
Bonferroni/Dunnett test was used to determine the significance of
differences in multiple comparisons. Values of P<0.05 were
categorized statistically significant.

Results
Initially, we examined the effect of cAMP analogues on the
number of human VSMCs under PDGF-stimulated conditions.
Treatment of human aortic VSMCs with recombinant PDGF (10
\text{ng/mL}) resulted in a significant increase in cell number, as
shown in Figure 1. Forskolin (30 \text{\mu M}) as well as 8-bromo-
cAMP (1 \text{\mu M}) and cilostazol (10^{-6} \text{ mol/L}), a stimulant of
cAMP and a PDE3 inhibitor) inhibited VSMC proliferation
stimulated by PDGF (vehicle 0.249±0.012, PDGF
0.279±0.015, PDGF+cilostazol 0.221±0.012 [P<0.01 versus
PDGF], PDGF+forskolin 0.187±0.006 [P<0.01 versus
PDGF], and 5\% serum 0.344±0.008). The inhibitory effect of
cAMP on VSMCs was examined in more detail by use of
cilostazol, a well-known cAMP stimulant used for the treatment
of peripheral arterial disease.\textsuperscript{4–6} Cilostazol inhibited
VSMC growth induced by PDGF in a dose-dependent manner (P<0.01,
Figure 1a). The inhibitory effect of cilostazol was still observed
at 2.5 and 10 ng/mL PDGF (P<0.01, Figure 1b). In contrast,
cilostazol did not alter basal growth of VSMCs in the absence of
PDGF. Recently, cell cycle regulation has been shown to be
important in the growth of VSMCs, in view of the fact that
inhibition of neointimal formation after angioplasty was
achieved by inhibition of cell cycle progression.\textsuperscript{21–24}
Therefore, we studied whether the inhibition of VSMC
proliferation by cilostazol was due to inhibition of the cell cycle.
Interestingly, cilostazol (10^{-6} \text{ mol/L}) reduced the number of
PCNA-positive–stained VSMCs, whereas the addition of PDGF significantly
increased PCNA-positive–stained VSMCs as assessed by flow

cytometry.

Given that cilostazol inhibited the growth of human
VSMCs at G_{1}/S phases, we have focused on the role of cell
cycle regulatory genes, especially on a negative regulator,
an anti-oncogene, p53. Decrease in p53 protein was
attenuated by cilostazol (10^{-6} \text{ mol/L}) as well as by forskol
in (30 \text{\mu M}) and 8-bromo cAMP (1 \text{mmol/L}), whereas
PDGF decreased p53 expression, as assessed by Western
blotting (Figure 2). To further confirm induction
of p53 protein by cAMP accumulation, we also measured
p21 protein, because p21 protein, which is induced by p53,
has been reported to be an important inhibitor of cell cycle
progression by inhibiting a complex of cdk kinases and
cyclins in various cells (including VSMCs) and also plays
a critical role in protecting cells against certain types of
injury.\textsuperscript{25,26} Upregulation of p33 protein by cAMP accumu
lation was further confirmed by the observation that the
decrease in p21 protein by PDGF was significantly atten
uated by cilostazol in a dose-dependent manner (Figure 3).
These results revealed that accumulation of cAMP inhibi
ted VSMC proliferation, which was at least in part due to
the increase in p53-p21 expression. Because p53 and p21
have been reported to induce apoptosis,\textsuperscript{27–29} we examined
apoptotic cells for cAMP accumulation. As shown in
Figure 4, we assessed the concordance between morpho
logical analysis and apoptosis assessed by nuclear staining
with Hoechst 33342. Cells treated with cAMP-inducing
agents exhibited the characteristic features of cell shrink
age, membrane blebbing, and rounding, which are typical of
apoptotic death, under phase-contrast microscopy. Simultaneous
assessment of nuclear chromatic morphology by
Hoechst 33342 staining verified that these cells eventually
manifested typical apoptotic condensed and coa-
lesced nuclei (Figure 4a). Incubation of VSMCs with cilostazol, compared with vehicle treatment, resulted in a significant increase in apoptotic cells in a dose-dependent manner; forskolin also stimulated apoptotic cells (Figure 4b). These results were confirmed by the measurement of DNA fragmentation (Figure 5). Consistent with nuclear staining, DNA fragmentation in VSMCs treated with forskolin as well as with 8-bromo cAMP and cilostazol was significantly increased compared with DNA fragmentation in VSMCs treated with vehicle, whereas PDGF significantly decreased the rate of DNA fragmentation ($P<0.01$, Figure 5). The increase in apoptosis by these agents was due to an increase in intracellular cAMP, because cAMP concentration was significantly increased by cilostazol as well as forskolin and 8-bromo-cAMP in a dose-dependent manner, even under PDGF stimulation (Figure 6). In addition, ($R$)-$p$-adenosine-$3',5'$-cyclic phosphorothioate (an inhibitor of the cAMP-dependent protein kinase) significantly attenuated the increase in apoptosis by cilostazol (data not shown).

**Discussion**

As discussed earlier, cAMP plays a pivotal role in the regulation of glucose metabolism, platelet aggregation, and dilatation of blood vessels. Therefore, accumulation of cAMP has potential therapeutic value in the treatment of peripheral vascular disease. Currently, many drugs that cause accumulation of cAMP have been developed to treat peripheral arterial disease.4–6 cAMP is synthesized from ATP by adenylate cyclase and converted to $5'\text{-AMP}$ by PDE.1 Prostaglandins I$_2$ and E directly activate adenylate cyclase, resulting in the accumulation of cAMP. In view of the fact that one of the rate-limiting steps in the cascade of the cAMP pathway is PDE, PDE inhibitors also inhibit platelet aggregation and VSMC relaxation through the activation of cAMP.2,3 Recently, a growing body of evidence has emerged implicating cAMP in the inhibition of VSMC migration.10,30–32 Specifically, studies using analogues of cAMP and activators of adenylyl cyclase have demonstrated that an increase in cAMP positively correlates with the inhibition of VSMC migration.30–32 Furthermore, downregulation of the major effector of cAMP, PKA, abrogates inhibition of VSMC chemotaxis by forskolin.10 Moreover, cAMP-mediated changes in cytoskeletal structures10,33 and alteration of gene expression via the cAMP response element binding protein may also be important.11,34 Activation of PKA by cAMP-elevating agents has been shown to attenuate VSMC proliferation, presumably by negatively modulating signaling by the mitogen-activated protein kinase pathway.12

![Figure 3](http://hyper.ahajournals.org/). Left, Typical example of Western blot of p21 and tubulin proteins in VSMCs treated with cilostazol. Untreat indicates VSMCs in serum-free medium; PDGF (2.5 ng/mL), PDGF (2.5 ng/mL)-stimulated VSMCs without cilostazol; and PDE3-i, PDGF (2.5 ng/mL)-stimulated VSMCs treated with cilostazol ($10^{-6}$ to $10^{-7}$ mol/L). Right, Percent change in ratio of p21 protein to tubulin protein in VSMCs treated with cilostazol. **$P<0.01$ vs Untreat; $#P<0.05$ and $$P<0.01$ vs PDGF.

![Figure 4a](http://hyper.ahajournals.org/). Typical example of apoptotic cells in VSMCs treated with cilostazol. No apoptotic change was observed in VSMCs treated with 2.5 ng/mL PDGF (normal cells). Morphologically apoptotic cells are shown with nuclear condensation, nuclear fragmentation, and merging after treatment with cilostazol. b, Number of apoptotic cells in VSMCs treated with cilostazol and forskolin. Untreat indicates VSMCs in serum-free medium; PDGF (2.5 ng/mL), PDGF (2.5 ng/mL)-stimulated VSMCs without cilostazol; PDE3-i, PDGF (2.5 ng/mL)-stimulated VSMCs treated with cilostazol ($10^{-6}$ to $10^{-7}$ M); and forskolin, PDGF (2.5 ng/mL)-stimulated VSMCs treated with forskolin (30 μmol/L). **$P<0.01$ vs Untreat; $#P<0.05$ and $$P<0.01$ vs PDGF. Values are expressed as percentage of apoptotic cells per total cell number. Each group contains 8 samples.
In addition to these actions, the present study revealed that VSMC growth was inhibited by the accumulation of cAMP, accompanied by upregulation of p53 (the p53 tumor suppressor gene) and p21 proteins by cAMP. p53 has been postulated to negatively regulate the cell cycle in some cell types. The presence of a functional p53 protein has been implicated as a critical determinant in the regulation of DNA replication, DNA repair, and programmed cell death. First, p53 has been shown to induce cell cycle arrest at the G1/S border, which is related to its ability to induce expression of a cellular gene, p21 (also known as WAF1/CIP1/SDI1), that encodes a 21-kDa inhibitor of G1 cyclin-dependent kinases. Second, p53 can induce apoptosis through bcl-2-dependent and -independent pathways. We also showed that p53 negatively regulated the cell cycle in human aortic VSMCs and was accompanied by apoptosis. Interestingly, the present study has demonstrated that accumulation of cAMP caused by forskolin and 8-bromo-cAMP, in addition to cilostazol, reverses the reduction in p53 protein by PDGF, as assessed by Western blotting. Reversal of reduction in p53 protein by cAMP accumulation was further confirmed by the observation that p21 was also increased by forskolin as well as 8-bromo cAMP and cilostazol. One of the probable mechanisms of cAMP in causing inhibition of VSMC growth is the induction of p53-p21. Indeed, we have also demonstrated that overexpression of p21 gene results in significant inhibition of neointimal formation in a vein graft model. Uptregulation of p53 and p21 proteins by cAMP would be important in considering the therapeutic value of cAMP-inducing agents, such as cilostazol, in view of the fact that dysfunction of p53 induced by cytomegaloviral infection was observed in patients with restenosis. Alternatively, cross talk between cAMP- and p53-generated signals has been reported in the induction of apoptosis in granulosa cells. Potential cross talk between cAMP and p53 might be related to the induction of apoptosis mediated by cAMP.

A fundamental pathological feature of vascular disease is the abnormal accumulation of cells within the intimal space, resulting in neointimal lesion formation produced by alterations in the homeostatic balance between cell growth and cell death. As mentioned earlier, p53 can induce apoptosis through bcl-2-dependent and -independent pathways. Recently, p53 has been reported to be a direct transcriptional activator of the bax gene, which is a homologous protein of the bcl-2 gene, but p53 has also been reported to attenuate bcl-2 function. Moreover, p21 has been reported to induce bax-dependent apoptosis in human VSMCs. Importantly, the present study has demonstrated a significant increase in apoptotic cells in VSMCs treated with cAMP stimulants (Figures 4 and 5). In view of the fact that VSMCs from atherosclerotic plaques showed a higher number of apoptotic cells than did normal VSMCs, induction of apoptosis by accumulation of cAMP may participate in the inhibition of VSMC growth.
growth in human subjects. Apoptosis mediated by cAMP is in line with the findings in a rat leukemia cell line, thymocytes, and primary granulosa cells. 45, 46

Overall, in the present study, we showed that cAMP has a direct inhibitory action against abnormal VSMC growth, accompanied by the induction of anti-oncogenes, p53 and p21, and apoptosis. These data suggest that p53/p21 may mediate the inhibitory effect of cAMP on VSMC proliferation induced by PDGF. Indeed, recent clinical studies have demonstrated that cilostazol reduces restenosis after angioplasty. 12–14 Agents that inhibit the cAMP-PKA signaling system may have potential clinical utility.

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