Cyclic Nucleotide Phosphodiesterase 1C Promotes Human Arterial Smooth Muscle Cell Proliferation

Sergei D. Rybalkin, Irina Rybalkina, Joseph A. Beavo, Karin E. Bornfeldt

Abstract—Proliferation of arterial smooth muscle cells (SMCs) is a key event in the formation of advanced atherosclerotic lesions and restenosis after angioplasty. Cyclic nucleotides (cAMP and cGMP) inhibit arterial SMC proliferation, and elevation of cyclic nucleotides reduces neointimal formation after angioplasty in animal models. Degradation of cAMP and cGMP is catalyzed by cyclic nucleotide phosphodiesterases (PDEs). One of these, PDE1C, hydrolyzes cAMP and cGMP and is expressed in proliferating human SMCs but is absent in quiescent human aorta. Thus, PDE1C expression is low in cultured human SMCs made quiescent by attaching to fibrillar collagen type I. After release from the fibrillar collagen, PDE1C expression is induced and associated with traverse through S-phase of the cell cycle. Further, PDE1C is expressed in vivo in human fetal aorta containing proliferating SMCs, but not in newborn aorta in which SMC proliferation has ceased. Inhibition of PDE1C in SMCs isolated from normal aorta or from lesions of atherosclerosis using antisense oligonucleotides or a PDE1 inhibitor results in suppression of SMC proliferation. In conclusion, PDE1C expression is a marker of human SMC proliferation ex vivo and in vivo. Inhibition of PDE1C leads to inhibition of human SMC proliferation. Because PDE1C is absent in quiescent SMCs, PDE1C inhibitors may target proliferating SMCs in lesions of atherosclerosis or restenosis. (Circ Res. 2002;90:151-157.)

Key Words: cAMP ■ cGMP ■ collagen ■ phosphodiesterase inhibitor

More than half of all deaths in the US is due to cardiovascular disease caused by atherosclerosis.1 This figure is increased to 70% to 80% for people with diabetes.2 Proliferation of arterial smooth muscle cells (SMCs) is a key event in the formation of advanced lesions of atherosclerosis3 and SMC proliferation is accelerated by diabetes.3 Therefore, considerable effort has been devoted to identification of factors that regulate SMC proliferation.

cAMP and cGMP inhibit proliferation of arterial SMCs.4,5 Intracellular levels of cAMP and cGMP are determined by the balance between their synthesis catalyzed by adenylyl- and guanylyl-cyclases and their degradation catalyzed by cyclic nucleotide phosphodiesterases (PDEs). PDEs are divided into at least 11 different gene families (PDE1 to 11) that together contain more than 50 different PDE isoenzymes.6,7 Of these, 3 are cAMP-specific (PDE4, PDE7, and PDE8), 5 degrade both cAMP and cGMP (PDE1, PDE2, PDE3, PDE10, and PDE11), and 3 isoforms are cGMP-specific (PDE5, PDE6, and PDE9).

PDEs in the PDE1 gene family are dependent on calcium-calmodulin (CaM) for activity and were previously termed CaM-PDEs. Three different PDE1 gene products have been cloned: PDE1A, PDE1B, and PDE1C. PDE1A and PDE1B hydrolyze cGMP more efficiently than cAMP, whereas PDE1C hydrolyses cAMP and cGMP with equal efficiency.6 Five PDE1C splice variants, PDE1C1 to 5, have been identified in human8 and mouse9 tissues.

In addition to activation by CaM, PDE1 can be regulated by induction of protein expression. Accordingly, expression of a PDE1 (most likely PDE1B) is markedly induced in proliferating lymphocytes.10,11 We have previously shown that normal human aorta does not express PDE1C in vivo, but that PDE1C is readily detected in SMCs cultured from the same aorta.12 Because placing SMCs in culture induces a switch from a quiescent phenotype to a proliferative phenotype, these observations suggested that induction of PDE1C may be required for degradation of the growth inhibitory cAMP and/or cGMP and for proliferation of SMCs.

Here we show that PDE1C expression is induced in proliferating human SMCs ex vivo and in vivo, and that inhibition of PDE1C activity results in an inhibition of SMC proliferation.

Materials and Methods

Cell Cultures and Tissues

Normal human diploid aortic SMCs were isolated from newborn aorta or the fibrous cap of lesions of atherosclerosis by the explant method.3 Cell cultures were kept in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 1% human plasma-derived serum (PDS) for 2 days before experiments. In some experiments, SMCs were plated onto fibrillar or monomer collagen type I (Vitrogen; Cohesion Technologies) according to a previously described method.13 The papillomavirus E6/E7-immortalized SMC line (FLTR) derived from human fetal aorta has been described previously.14 Cell...
cultures were used for experiments or plating onto collagen-coated plates when near-confluent.

Human fetal aortas, gestational age 74 to 145 days, were obtained from the Central Laboratory for Human Embryology at the University of Washington. A segment of each aorta was fixed in methyl Carnoy’s fixative for immunohistochemistry. The remaining aorta specimen was stripped from fat and adventitia, and the endothelial cell layer was scraped off. The smooth muscle layer was pulverized under liquid nitrogen using a pestle and mortar, and then transferred into a buffer containing 50 mmol/L β-glycerophosphate (pH 7.4), 1.5 mmol/L EGTA, 0.1 mmol/L Na3VO4, 1 mmol/L DTT, 10 μg/mL aprotinin, 5 μg/mL pepstatin, 20 μg/mL leupeptin, and 1 mmol/L benzamidine. The tissue powder was homogenized using a Polytron homogenizer (Brinkmann Instruments, Inc) followed by sonication (two 10-second pulses) using a Braun-Sonic sonicator (B. Braun Biotech Inc) at 50% output. Tissue extracts were centrifuged at 100 000g for 20 minutes at 4°C, and the supernatant was used for analysis of PDE1C expression and activity.

Reagents
Human recombinant PDGF-BB was from Upstate Biotechnology, Inc (Lake Placid, NY). C-type natriuretic peptide (CNP 1 to 22) was dissolved in DMSO at a stock concentration of 30 mmol/L. Anti-inhibitor 3-isobutyl-1-methylxanthine (IBMX). Sildenafil (Viagra) (PDE5 inhibitor), and the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (BMX). Sildenafil (Viagra) was a generous gift from Pfizer Inc. All PDE inhibitors were dissolved in DMSO at a stock concentration of 30 mmol/L. Anti-PDE1C antibodies and recombinant murine PDEs were generated as described previously.12

Western Blot Analysis and Immunohistochemistry
Western blot analyses of PDE1C and PDE5 were performed using isoezyme-specific antibodies.12 Immunohistochemical identification of SMCs expressing proliferating cell nuclear antigen (PCNA) was done by double-staining,3 using a mouse monoclonal anti-smooth muscle α-actin antibody (DAKO Corp, Carpinteria, Calif), a mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), and purified mouse IgG2a or IgG2b as negative controls (Zymed Labs Inc, South San Francisco, Calif). The negative controls did not result in staining of the specimens.

PDE Activity Measurements and Effects of PDE Inhibitors
PDE activity measurements were performed using 1 μmol/L cAMP or cGMP as substrate, as described previously.12 To determine the ability of pharmacological PDE inhibitors to inhibit cAMP PDEs (PDE1C and PDE412) in human SMCs, PDEs were partially purified using HPLC on MonoQ quaternary ammonium strong anion exchange HR 5/5 columns (Amersham Pharmacia Biotech Inc), as described previously.12 Peak fractions containing PDE1C or PDE4 were used for inhibition studies. We were unable to sufficiently purify PDE3 from human SMCs using anion exchange HPLC. However, because human SMCs express both PDE3A and PDE3B13 and there are no known differences in the ability of the inhibitors used to inhibit PDE3A and PDE3B, recombinant murine PDE3B was used to evaluate inhibition of PDE3. For each PDE inhibitor, the concentration required to give half-maximal inhibition (IC50) of PDE activity was determined and calculated using GraphPad Prism 2.0.

Measurement of cAMP and cGMP Levels
Immediately after stimulation of SMCs with the indicated concentrations of prostaglandin E2 (PGE2; Biomol) to elevate cAMP levels, CNP to elevate cGMP levels and PDE inhibitors, the cells were washed twice in ice-cold PBS and lysed in 70% ice-cold ethanol. Intracellular levels of cAMP and cGMP were measured using Biotrak enzyme assay kits (Amersham).16 Levels of cAMP were measured in nonacyetylated samples whereas samples for cGMP determination were acetylated.

PDE1C Antisense Oligonucleotide Studies
Reverse transcription–polymerase chain reaction (RT-PCR) was used to determine part of the sequence, starting at the ATG site, of the PDE1C variant expressed in human SMCs. Primers were synthesized (GIBCO-BRL) using the published sequence of human PDE1C1 (forward 5′-ATGGAGTGCCAAACCAAGG-3′; reverse 5′-TTGGATGACTGGAACCATAG-3′). RNA was purified,13 and the resulting RT-PCR product was sequenced. The product showed a 100% identity with the sequence of PDE1C1 and PDE1C3.8 We did not distinguish between the PDE1C1 and PDE1C3 splice variants, which differ only in their 5′ end.8 However, based on the apparent SDS gel molecular weight (~73 kDa), the splice variant expressed in human SMCs is most likely either PDE1C1 or the human equivalent of mouse PDE1C4.9

PDE1C antisense oligonucleotides were designed by using the cloned PDE1C sequence, melting temperature, and predicted secondary structures. Phosphothioate PDE1C antisense oligonucleotides (tissue culture grade) corresponding to the nucleotide sequence 171 to 187 (ACTCGATACTCTAGCCG) and reversed control (GGG-GACTCTCATGCTCA) oligonucleotides were synthesized by Oligo Etc, Inc. FLTR cells were plated onto fibrillar collagen for 2 days to reduce basal expression of PDE1C. The cells were then replated onto tissue culture plates in DMEM/1% PFS for 15 hours. Lipofectamine (GIBCO-BRL) was used for delivery of oligonucleotides (10 μmol/L) into cells. The transfection was performed in serum-free DMEM for 8 hours, and the medium was then replaced with DMEM containing 10% FBS. Although control phosphothioate oligonucleotides inhibited SMC proliferation by ~40% through a nonspecific mechanism, the protocol used resulted in specific effects of PDE1C antisense oligonucleotides on PDE1C expression. Following a subsequent 3-day incubation, PDE1C expression was measured using Western blot analysis, and SMC number was measured by counting.3

Measurement of DNA Synthesis and SMC Proliferation
Measurements of DNA synthesis and cell number were performed as described previously.3

Statistical Analysis
Statistical analysis was performed using 1-way ANOVA followed by the Newman-Keuls multiple comparison test or using 2-tailed unpaired t test for comparison of 2 groups. Levels of significance are denoted as **P<0.01 and ***P<0.001.

Results
PDE1C Is Induced in Proliferating Human SMCs in Culture
We have shown before that PDE1C is absent in intact human aorta but is readily detected in cultured SMCs from the same donor.12 Based on this information, we postulated that PDE1C expression may be induced in proliferating SMCs. To make cultured SMCs quiescent, the cells were plated onto fibrillar collagen type I, which results in cell cycle arrest13,17 as shown by Figure 1A, human SMCs plated onto fibrillar collagen for 2 days show significantly reduced PDE activity in fractions 4 to 12 following separation of cell extracts on anion exchange HPLC columns. The CaM-stimulated PDE activity in these fractions is due to PDE1C.12 There is a slight increase in CaM-independent cAMP hydrolyzing activity (fractions 28 to 36), previously identified to be due to PDE3 and PDE4.4

The reduced PDE1C activity in SMCs plated onto fibrillar collagen for 2 days correlates with a reduction of PDE1C activity.
protein expression (Figure 1B). When these SMCs are released from the collagen and replated onto uncoated plates, PDE1C expression (Figure 1B) and activity (data not shown) are markedly induced.

Because normal human SMCs express PDE3 and PDE4 that contribute to total cAMP hydrolyzing activity, we took advantage of an immortalized fetal aortic SMC line (FLTR). The PDE expression profile in these cells is similar to that of human SMCs isolated from lesions of atherosclerosis in that these cells express abundant PDE1C (fractions 3 to 15), but little or no PDE3 or PDE4 (Figure 2A). PDE1C is the only detectable CaM-PDE in these cells. When these SMCs were plated onto fibrillar collagen there was a nearly 90% reduction of CaM-PDE (PDE1C) activity (Figure 2B). No detectable differences in other CaM-independent PDE activities were observed (Figure 2B).

Next, we investigated whether induction of PDE1C expression correlates with induction of proliferation in FLTR cells released from fibrillar collagen. Thymidine incorporation into DNA and PDE1C protein levels were measured at different times after release from fibrillar collagen. Half-maximal DNA synthesis was achieved after a 21-hour incubation in 10% FBS (Figure 3). There was a correlation between induction of DNA synthesis and induction of PDE1C expression (Figure 3). Interestingly, SMCs that had not previously been exposed to fibrillar collagen showed a half-maximal induction of DNA synthesis at 15 hours (Figure 3). These results agree with our observations that SMCs become more quiescent when plated onto fibrillar collagen compared with plastic or monomeric collagen. Thus, induction of PDE1C correlates with cell cycle progression in human SMCs.

**PDE1C Is Expressed in Proliferating Human Arterial Tissue In Vivo**

To investigate whether PDE1C is expressed in proliferating SMCs in vivo, human fetal aortas were double stained using the SMC marker smooth muscle α-actin and PCNA as a marker of cells that have entered the cell cycle. PDE1C expression in the smooth muscle layer was measured using Western blot analysis. Two PDE1C isoforms with apparent molecular weights of 73 kDa and 75 kDa are present in the human fetal aorta, whereas cultured SMCs only express the 73 kDa (PDE1C1 or PDE1C4) isoform. Human fetal aorta shows abundant numbers of PCNA-positive SMCs at a gestational age of 74 days and at 53 to 127 days (data not shown). Human newborn aorta did not show detectable expression of PDE1C (Figure 4A), consistent with the lack of SMC proliferation in this tissue (Figure 4C). Thus, PDE1C is expressed in proliferating human SMCs in fetal aorta in vivo.
Inhibition of PDE1C Reduces SMC Proliferation

In order to investigate the role of PDE1C in SMC proliferation, we used 2 approaches, namely pharmacological inhibitors and antisense oligonucleotides. Because completely specific small-molecule PDE1C inhibitors are unavailable, we measured the effects of various PDE inhibitors on PDE1C and other PDEs expressed in human SMCs (Table). We have previously shown that the PDE isozymes detectable by HPLC in human SMCs are PDE1C, PDE3, PDE4, and PDE5.12 These PDEs represent the total PDE activity measured at 1 μmol/L substrate concentration. As shown by the Table, the PDE1 inhibitor 8MM-IBMX inhibits PDE1C with an IC50 value of 12 μmol/L, whereas 303 μmol/L is required for PDE3B and 135 μmol/L is required for PDE4. Thus, 8MM-IBMX exhibits an ∼11-fold and ∼25-fold specificity for PDE1C over PDE4 and PDE3, respectively. The PDE3 inhibitor enoximone and the PDE4 inhibitor rolipram do not inhibit PDE1C at concentrations below 100 μmol/L (Table). The PDE5 inhibitor zaprinast inhibits PDE1C with an IC50 value of 10 μmol/L. The nonselective PDE inhibitor IBMX inhibits all PDEs, as expected. Thus, 8MM-IBMX at 10 to 30 μmol/L can be used to selectively inhibit PDE1C over other cAMP-PDEs in human arterial SMCs. Although 8MM-IBMX can inhibit PDE5 at concentrations ∼30 μmol/L, PDE5 inhibitors do not mimic the effects of 8MM-IBMX, indicating that the effects of 8MM-IBMX are not due to PDE5 inhibition.

With this information at hand, we investigated effects of 8MM-IBMX on SMC proliferation. As shown in Figure 5A, 8MM-IBMX at PDE1C-selective concentrations significantly inhibits DNA synthesis in normal human SMCs. 8MM-IBMX also results in increases in both cAMP and cGMP in the absence or presence of inhibitors of other PDEs (Figure 5B). Because PDE1C hydrolyzes both cAMP and cGMP, these findings further support the observation that PDE1C is the principal target of 8MM-IBMX. The PDE5 inhibitors zaprinast and sildenafil have little effect on DNA synthesis under the conditions tested at concentrations of 30 and 1 μmol/L, respectively (data not shown). However, inhibition of PDE3 or PDE4 by 10 to 30 μmol/L enoximone and rolipram, respectively, results in a significant inhibition of DNA synthesis in normal SMCs (data not shown), as has been reported previously.4,13–23 Inhibition of PDE1C, PDE3, PDE4, and PDE5 using the nonselective PDE inhibitor IBMX (Figure 5A) or a combination of 8MM-IBMX, enoximone, and rolipram (data not shown) gives a comparable inhibition of DNA synthesis in these cells.

In contrast to normal SMCs, which express relatively high levels of PDE3 and PDE4, SMCs derived from human atherosclerotic lesions express little PDE3/4; PDE1C there-
fore accounts for nearly 100% of the total cAMP-hydrolyzing activity in these cells. Accordingly, the PDE3 inhibitor enoximone and the PDE4 inhibitor rolipram are relatively poor inhibitors (20% to 30% inhibition) of DNA synthesis in lesion SMCs (Figure 5C). Inhibition of PDE1C by 8MM-IBMX, on the other hand, results in an approximate 50% to 60% inhibition of DNA synthesis at 10 to 100 \( \mu \text{mol/L} \). These results indicate that PDE1C activity is required for maximal proliferation of SMCs isolated from the normal aorta and from lesions of atherosclerosis.

Because 8MM-IBMX does not exhibit more than a \( \approx 11\)-fold specificity for PDE1C over PDE4, we also inhibited PDE1C by antisense oligonucleotides. Because the turn-over rate of PDE1C is \( \approx 24\) hours (data not shown), and basal PDE1C expression in SMCs is high, we plated SMCs onto fibrillar collagen for 2 days to down-regulate PDE1C expression and then replated the cells onto uncoated tissue culture plates. The blot shows a lack of PDE1C induction in FLTR cells treated with PDE1C antisense oligonucleotides and an induction of PDE1C expression in cells treated with the control oligonucleotide (reversed). The density of the PDE1C band in A was analyzed. Control (cells on fibrillar collagen) was set to 100%. C, Three days after replateing, FLTR cells treated with PDE1C antisense oligonucleotides or control oligonucleotides were counted. Control (cells on fibrillar collagen) was set to 100%. The results are shown (B and C) as mean \( \pm \) SD of representative experiments. The experiment was repeated 3 times. Asterisks show statistically significant differences between the groups (t test).

PDE1C Is Induced in Proliferating SMCs Ex Vivo and In Vivo

We show that PDE1C expression is a marker of human SMC proliferation ex vivo and in vivo. It is likely that PDE1C is induced in the transition from a quiescent cell in G0 of the cell cycle to G1 and S. This concept is supported by a number of observations. First, PDE1C is not expressed in intact human aorta, which consists of quiescent SMCs, but is highly expressed in SMCs isolated from the same donor and stimulated to proliferate in culture. Second, human fetal aorta, which contains abundant SMCs in the cell cycle, expresses PDE1C in the smooth muscle layer. Third, SMCs plated onto fibrillar collagen have a markedly reduced PDE1C expression. This procedure results in complete growth arrest and a phenotype similar to SMCs in the intact aorta. Fourth, there is a clear correlation between induction of DNA synthesis and induction of PDE1C expression in SMCs that have been released from fibrillar collagen. Interestingly, SMCs on uncoated dishes or on monomeric collagen retain PDE1C expression under low serum conditions, and S-phase entry in these cells is not delayed, as is the case for SMCs released from fibrillar collagen. Taken together, these observations suggest that either complete quiescence or exit of SMCs from the cell cycle into G0 is required to reduce PDE1C expression. It is therefore tempting to speculate that PDE1C activity is necessary for normal entry into G1- and subsequent S-phase.

PDE1C Promotes SMC Proliferation: Is the Effect Mediated by Decreased Levels of cAMP or cGMP?

We show that PDE1C activity is required for maximal proliferation of human SMCs by the use of pharmacological PDE inhibitors at concentrations that are selective for PDE1C under the conditions used and by the use of PDE1C antisense oligonucleotides. Therefore, one of the normal functions of PDE1C is to promote SMC proliferation. It is likely that the effect of PDE1C on proliferation is due to its ability to hydrolyze cAMP. Although cAMP and cGMP both are known to inhibit SMC proliferation, endogenous intracellular cGMP levels are 10- to 1000-fold lower than those of cAMP in human SMCs (Figure 5B). Interestingly, it has recently been shown that whereas a cAMP analog leads to cell cycle arrest in SMCs, a cGMP analog at the same concentration delays, but does not block, cell cycle traverse.

Furthermore, inhibition of cAMP PDEs result in a much greater inhibition of SMC proliferation than inhibition of cGMP PDEs. For example, the PDE5 inhibitors zaprinast and sildenafil give only a weak or no inhibition of SMC DNA synthesis under conditions where cGMP synthesis is stimulated with CNP, despite the high expression of PDE5 in human SMCs (data not shown). In fact, a recent study shows that PDE5 inhibitors reduce SMC proliferation via cGMP-induced inhibition of PDE3 and subsequent elevation of cAMP. PDE1C is not the only cAMP PDE in SMCs; thus, inhibition of PDE4, and to a lesser extent of PDE3, also reduces DNA synthesis in SMCs, as has been previously reported.

The growth inhibitory action of cAMP in SMCs is most likely due to activation of cAMP-dependent protein kinase (PKA) and subsequent suppression of several mitogenic signal transduction pathways. For example, elevation of cAMP leads to inhibition of the extracellular-signal regulated kinase (ERK) pathway in SMCs. cAMP-elevating agents also inhibit activity of the phosphatidylinositol 3-kinase (PI3K) pathway. The target of cAMP in this pathway awaits identification, but may be located upstream of PI3K. In addition to these acutely activated mitogenic signaling pathways, cAMP can prevent activation of cyclin-dependent kinases. Therefore, it seems likely that degradation of cAMP accounts for PDE1C’s growth-promoting effects.

PDE Inhibitors as SMC Growth Inhibitors

It has been shown that elevation of cAMP reduces formation of neointimal lesions and inhibits SMC proliferation after arterial injury in animal models. In one study, the nonselective PDE inhibitor aminophylline and the PDE3-selective inhibitor amrinone were found to have similar effects. Other PDE3 inhibitors have also been shown to inhibit neointimal thickening after endothelial injury. As shown in the present and previous studies, PDE3 and PDE4 inhibitors are effective in inhibiting proliferation of normal SMCs. Our studies show that SMCs isolated from lesions of atherosclerosis have very low levels of PDE3 and PDE4 and that PDE3 and PDE4 inhibitors only marginally affect proliferation in these cells. Because PDE3 and PDE4 isozymes are present in the normal arterial wall as well as in many other tissues, inhibition of these PDEs is likely to cause side-effects, such as vasodilatation, nausea, and cardiac arrest. PDE1C is not expressed in normal quiescent arterial SMCs or in human endothelial cells isolated from the umbilical vein (S.D. Rybalkin, I. Rybalkina, J.A. Beavo, K.E. Bornfeldt; unpublished observations, 2001). In contrast, PDE1C is likely to be induced in proliferating human SMCs after angioplasty or in lesions of atherosclerosis, which may allow for selective targeting of these cells in vivo. Indeed, our studies on human SMCs isolated from lesions of atherosclerosis show that PDE1C is the principal cAMP PDE in these cells. Interestingly, PDE1C is not induced in proliferating SMCs from any other species studied to date, which makes animal models commonly used for studies of vascular injury less useful for studies of PDE1C, and perhaps of PDE inhibitors in general. Thus, evaluation of the role of PDE1C in SMC proliferation and accumulation in the injured human arterial wall is dependent on the development of highly selective PDE1C inhibitors or gene therapies. Future studies will reveal if PDE1C can indeed be targeted to inhibit human SMC proliferation in restenosis after angioplasty, in-stent restenosis, or in lesions of atherosclerosis.

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