Prostacyclin Analogues Differentially Inhibit Growth of Distal and Proximal Human Pulmonary Artery Smooth Muscle Cells

John Wharton, PhD; Neil Davie, BSc; Paul D. Upton, PhD; Magdi H. Yacoub, FRS; Julia M. Polak, FRCPath; Nicholas W. Morrell, MD

**Background**—Prostacyclin has proved to be a beneficial treatment for patients with severe pulmonary hypertension. We postulated that the response may reflect, at least in part, inhibition of pulmonary artery smooth muscle cell (PASMC) growth.

**Methods and Results**—Human PASMCs were derived from distal (<1-mm external diameter, n=8) and proximal (>8-mm external diameter, n=12) pulmonary arteries obtained at transplant surgery and pneumonectomy. The effects of the stable prostacyclin analogues on [methyl-3H]thymidine incorporation and cell proliferation were investigated by using immunohistochemically characterized cells. Distal cells proliferated faster than did proximal PASMCs and displayed a distinct sensitivity to cicaprost and iloprost. Both analogues inhibited thymidine uptake over 24 hours (20% to 60%, P<0.001; n=8) and abolished stimulation of DNA synthesis by platelet-derived growth factor-BB (10 ng/mL) in distal but not proximal cells. The inhibitory effect of cicaprost was mimicked by isoproterenol (10^{-5} mol/L), forskolin (10^{-5} mol/L), and dibutyryl cAMP (5×10^{-4} mol/L) and was potentiated by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (5×10^{-2} mol/L). Cicaprost (10^{-9} to 10^{-6} mol/L) inhibited the proliferation of PASMCs, which had been stimulated with either platelet-derived growth factor-BB or serum, and increased cAMP production. These effects were potentiated by 3-isobutyl-1-methylxanthine and attenuated by the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (10^{-3} to 10^{-4} mol/L).

**Conclusions**—Cicaprost and iloprost inhibit DNA synthesis and proliferation to a greater extent in distal compared with proximal human PASMCs, acting at least in part via a cAMP-dependent mechanism. The results are consistent with the hypothesis that prostacyclin analogues inhibit vascular remodeling in pulmonary hypertension and demonstrate heterogeneity among human PASMCs. *(Circulation. 2000;102:3130-3136.)*

**Key Words:** prostaglandins ■ hypertension, pulmonary ■ muscle, smooth ■ remodeling

Prostacyclin (PGI\textsubscript{2}) is the main arachidonic acid metabolite in the mammalian vasculature, with production being greater in pulmonary than systemic arterial segments.\textsuperscript{1} It is produced by PGI\textsubscript{2} synthase and is a powerful vasodilator of both systemic and pulmonary vascular beds.\textsuperscript{2} Deficiencies and perturbation of PGI\textsubscript{2} production have been associated with pulmonary hypertension in humans and experimental animals. Patients with severe pulmonary hypertension have an imbalance in the local production of vasoactive eicosanoids\textsuperscript{3} and reduced pulmonary artery expression of PGI\textsubscript{2} synthase,\textsuperscript{4} whereas overexpression of the enzyme protects mice against hypoxic pulmonary hypertension.\textsuperscript{5}

Most important, long-term infusion of PGI\textsubscript{2}, or its stable analogue iloprost, improves survival and reduces pulmonary vascular resistance in patients with either primary\textsuperscript{6,7} or secondary\textsuperscript{8,9} pulmonary hypertension. This is not surprising, inasmuch as characteristic changes in vascular structure are common to both forms of the disease; muscular arteries and arterioles show smooth muscle proliferation, medial hypertrophy, the development of distal musculature, and fibrosis.\textsuperscript{10,11}

PGI\textsubscript{2} infusion is not only an important treatment for severe pulmonary hypertension, but it is also effective in patients who do not exhibit acute vasodilatation,\textsuperscript{6} suggesting that effects other than pulmonary vasodilatation contribute to the therapeutic response. Mechanisms examined to date include normalization of the prothrombotic state\textsuperscript{12} and improved balance between endothelin-1 clearance and release\textsuperscript{13}; however, PGI\textsubscript{2} could also directly affect hypertensive pulmonary artery remodeling by controlling cell proliferation.

Vasodilatory prostaglandins and PGI\textsubscript{2} analogues generally inhibit the growth of systemic vascular smooth muscle cells...
(SMCs). Gene transfer of PGI₂ synthase has also been reported to inhibit systemic vascular remodeling in vivo. In contrast, the effects of PGI₂ and its analogues on pulmonary artery SMC (PASMCS) growth are less certain, with vasodilatory prostaglandins having been found to stimulate rather than inhibit the proliferation of bovine PASMCS. Therefore, we sought to establish whether the stable PGI₂ analogues iloprost and cicaprost could influence the growth of human PASMCs derived from distal and proximal regions of the pulmonary artery tree.

Methods

Reagents

DMEM (high glucose), collagenase type II, agarose (36°C to 42°C gelling temperature), antibiotic-antimycotic solution, trypsin-EDTA, and 8-well chamber slides were from Life Technologies. Accutase, SM medium, and growth supplement (containing insulin, human epidermal growth factor, human fibroblast growth factor, and 5% FBS) were purchased from TCS Biologicals Ltd. Unless otherwise stated, other reagents were obtained from Sigma-Aldrich Co Ltd.

Isolation of PASMCs

Human lung and segments of pulmonary artery (trunk or right/left lobar) were obtained at lung or heart-lung transplantation from patients (3 males and 4 females, mean age 43.0 years) with primary pulmonary hypertension (n = 2), congenital heart disease (n = 1), emphysema (n = 1), obliterative bronchiolitis (n = 1), sarcoidosis (n = 1), or fibrosing alveolitis (n = 1). Pulmonary artery samples were collected from unused donor tissues (5 males and 2 females, mean age 36.4 years). Further lung specimens were obtained at lobectomy or pneumonectomy for bronchial carcinoma (2 males and 4 females, mean age 65.5 years). Ethical approval was obtained from Hammer smith and Harefield Hospital ethics committees.

Distal PASMCs were isolated from peripheral segments of artery (<1-mm external diameter) after either microdissection or magnetic separation. The latter involved infusing a warmed suspension of iron oxide particles (0.5% [wt/vol]) in DMEM and agarose (0.5% [wt/vol]) into a lobar pulmonary artery and bronchus. Once the agarose had set, subpleural strips of lung were dissected, large vessels were removed, and the remaining tissue was minced and digested with collagenase (1000 U/mL) for 4 hours at 37°C. The parenchyma was further disrupted by shearing, and vessels were isolated by using a magnetic concentrator (Dynal UK Ltd) before plating in DMEM containing 20% FBS. Intact lung lobes were rarely available; therefore, most distal PASMCs were obtained after microdissection. Arterial segments (0.3- to 1.0-mm external diameter) and attached branches were separated from the parenchyma and adventitia, washed in PBS, minced, and digested in collagenase (1000 U/mL) for 4 hours at 37°C. The suspension was drawn through a 1-mL pipette at hourly intervals, filtered (100-m pores), and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in SM medium—growth supplement and plated in 6- to 12-well culture plates. The medium was changed every 2 days, and adherent cells were dissociated by using Accutase.

Proximal PASMCs were obtained from trunk and lobar arteries (>8-mm external diameter), either from explants or after digestion in collagenase, as described. Irrespective of their origins, PASMC isolates (passages 3 and 10) were initially plated in DMEM containing 10% FBS for all experiments.

Serum-Induced Growth and Phenotypic Characterization of PASMCs

Distal and proximal PASMCs were seeded at 1.5 × 10⁴ cells per well in DMEM containing 10% FBS in 24-well plates. The medium was replaced every 48 hours, and the cells were counted at intervals with use of a hemocytometer. The SMC phenotype was confirmed immunohistochemically, as described, by use of monoclonal antibodies to vimentin (clone V9), α-smooth muscle actin (clone IA4), and antiserum to smooth muscle myosin, provided by Dr Frid, University of Colorado Health Science Center, Denver. The presence of fibroblast and endothelial cell phenotypes was excluded by use of antibodies to human fibroblast surface protein (clone IB10), proline-4-hydroxylase (clone 5B5), and von Willebrand factor (A0082, Dako Ltd). Nuclei were counterstained with 4,6-diamidino-2-phenylindoline (0.01 μg/mL in PBS for 1 minute) or Sytox Green (1 μmol/L in H₂O for 5 minutes; Molecular Probes).

Effect of PGI₂ Analogues on DNA Synthesis

DNA synthesis was measured by [methyl-3H]thymidine incorporation over 24 hours. Cells were suspended in DMEM containing 10% FBS, seeded in 24-well plates (10⁴ cells per well), grown until 80% to 90% confluent, and brought to quiescence by incubation in serum-free DMEM for 2 hours followed by serum deprivation (DMEM containing 0.1% FBS) for 72 hours. Cells were then incubated in fresh medium containing 0.25 μCi per well [methyl-3H]thymidine (Amersham Pharmacia Biotech Ltd). The effect of PGI₂ analogues was determined by adding 10⁻¹⁰ to 10⁻⁹ mol/L of either cicaprost (Schering) or iloprost (Amersham Pharmacia), with or without platelet-derived growth factor (PDGF)-BB (10 ng/mL) or 10% FBS. In additional experiments, cicaprost was added for most (20 to 24 hours) or part (last 4 to 6 hours) of the total incubation period.

The roles of adenylyl cyclase and cAMP production were investigated by adding 3-isobutyl-1-methylxanthine (IBMX, 5 × 10⁻⁵ mol/L), dibutyryl cAMP (dbcAMP, 5 × 10⁻⁴ mol/L), forskolin (10⁻⁵ mol/L), or isoprotanol (10⁻³ mol/L). Adenylyl cyclase inhibitors 2',5'-dideoxyadenosine (2',5'-DDA) and SQ-22536 (Calbiochem-Novabiochem Ltd) were added (10⁻⁴ to 10⁻⁵ mol/L) 1 to 2 hours before, as well as during, stimulation of PASMCs. The [methyl-3H]thymidine content of cell lysates was determined by scintillation counting, as described.

Effects of Cicaprost on Cell Cycle, Proliferation, and cAMP Production

To determine the effect of cicaprost on cell cycle distribution, subconfluent distal PASMCs were brought to quiescence and incubated for a further 24 hours, with or without cicaprost (10⁻⁷ mol/L), in DMEM containing 0.1% FBS alone or in medium supplemented with PDGF-BB (10 ng/mL). Cells (1 to 2 × 10⁶) were resuspended in 0.5 mL ice-cold PBS, fixed in ice-cold ethanol (1.0 mL), washed in PBS, and incubated in RNase (50 μg/mL PBS) for 30 minutes at 37°C. Propidium iodide (50 μg/mL PBS) was added, and the DNA content was evaluated by flow cytometry (FACS Vantage, Becton Dickinson). The effect of cicaprost on distal PASMC proliferation was determined during stimulation with either PDGF-BB (10 ng/mL) or 10% FBS. Cells were seeded in 24-well plates (10⁴ cells per well), cultured for 48 hours in DMEM containing 10% FBS, brought to quiescence, and stimulated with or without cicaprost and/or 2',5'-DDA. Medium was changed on alternate days, and cells were counted with a hemocytometer.

Intracellular cAMP accumulation was determined by using distal PASMCs grown to confluence in 24-well plates. Cells were incubated for 3 hours in serum-free DMEM and stimulated with either cicaprost (10⁻¹⁰ to 10⁻⁶ mol/L), forskolin (10⁻⁵ mol/L), or isoprotanol (10⁻⁵ mol/L) for 15 minutes at 20°C to 25°C in the absence or presence of 5 × 10⁻⁵ mol/L IBMX and the adenylate cyclase inhibitors SQ-22536 and 2',5'-DDA. The time course (0 to 4 hours) of cicaprost-stimulated (10⁻⁷ mol/L) cAMP production was also determined at 37°C in the absence of IBMX. After stimulation, cells were extracted in 250 μL acid ethanol (75% ethanol, 16 mmol/L HCl), and dried extracts were assayed for cAMP by using a commercial ¹²⁵I-labeled cAMP assay (NEN, Life Science Products Inc).

Statistical Analysis

Data were expressed as mean±SEM and analyzed with GraphPad Prism version 3.0 (GraphPad Software). Comparisons were made by
Eight distal PASMC cultures were derived, each from separate individuals (aged 52.3±1.6 SEM years). Proximal PASMC cultures were obtained from 12 patients (44.3±5.0 SEM years). Cells isolated from distal regions and cultured in the presence of 10% FBS appeared stellate or spindle-shaped, forming irregular clusters and, later, sheets of cells (Figure 1A and 1B). When confluent and after quiescence, they formed continuous sheets of elongated bipolar cells arranged in parallel. No endothelial or fibroblast cells were identified, and the smooth muscle phenotype was confirmed by vimentin, α-smooth muscle actin, and smooth muscle myosin immunofluorescence staining (Figures 1C through 1E). The phenotype of proximal PASMC isolates has been previously described: the cells have been reported to exhibit immunohistochemical characteristics similar to those of distal PASMCs. However, the distal PASMCs exhibited a higher rate of growth than did cells isolated from large elastic pulmonary arteries (Figure 2); doubling time for distal PASMCs (3.2±0.3 days, n=6) was significantly shorter (P=0.0005) than that for proximal cells (11.1±1.46 days, n=7).

**Effect of PGI<sub>2</sub> Analogues on DNA Synthesis**

Cicaprost and iloprost induced concentration-dependent (10⁻⁸ to 10⁻⁷ mol/L) inhibition of thymidine uptake in distal PASMCs in the presence of either DMEM containing 0.1% FBS alone or stimulated with 10 ng/mL PDGF-BB (Figure 3A and 3B). In contrast, DNA synthesis in proximal PASMCs maintained in DMEM containing 0.1% FBS was not inhibited by the PGI<sub>2</sub> analogues, and the inhibitory effect on PDGF-BB–stimulated thymidine uptake was relatively weak (Figure 3C). The differential response occurred despite a comparable stimulation of DNA synthesis by PDGF-BB, with the thymidine uptake being increased 3- to 4-fold over 24 hours in proximal (3.1±0.7, n=5) and distal (4.0±0.9, n=8; P=0.499) PASMCs without any change in cell number. These initial findings indicated that cicaprost and iloprost had similar effects on DNA synthesis, but in view of the specificity of cicaprost as an agonist for the prostaglandin IP receptor subtype, it was used in subsequent experiments.

The inhibitory effect of cicaprost (10⁻⁷ mol/L) on DNA synthesis was significantly greater (P<0.0001) when added to distal PASMCs for most (20 to 24 hours) rather than part (last 4 to 6 hours) of the incubation period, irrespective of whether the cells were maintained in DMEM containing 0.1% FBS (22.0±1.3% versus 64.7±9.1% of control) or stimulated with 10 ng/mL PDGF-BB (7.9±0.2% versus 60.1±3.2% of control, n=3). The inhibitory effect of PGI<sub>2</sub> analogues on DNA synthesis was mimicked by either isoproterenol (10⁻⁵ mol/L), forskolin (10⁻⁷ mol/L), or dbcAMP (5×10⁻⁴ mol/L) (Figure 4A). Both cicaprost (10⁻⁷ mol/L) and forskolin (10⁻⁵ mol/L) induced significantly greater inhibition of thymidine uptake in PDGF-BB–stimulated distal PASMCs than in proximal cells (Figure 4B). The addition of IBMX (5×10⁻⁵ mol/L) inhibited DNA synthesis in distal PASMCs and potentiated the effect of cicaprost, causing significantly greater inhibition of [methyl-³H]thymidine uptake (Figure 4C). DNA synthesis in proximal cells was also inhibited by coincubation with IBMX and 10⁻⁷ mol/L cicaprost (57.2±2.9% of control, n=4) but to a lesser extent (P=0.003) than that observed in distal PASMCs (23.3±5.9% of control, n=7).

The addition of adenylyl cyclase inhibitors (10⁻⁵ mol/L 2',5'-dideoxycytidine and 10⁻⁴ mol/L SQ-22536) had no significant effect on DNA synthesis over 24 hours, and the presence of 2',5'-dideoxycytidine at a higher concentration (10⁻³ mol/L) reduced both cell viability and thymidine incorporation (data not shown).
Effects of Cicaprost on Cell Cycle, Proliferation, and cAMP Production

Cicaprost inhibited the progression of distal PASMCs from G0/G1 to the S phase of the cell cycle (Figure 5) and inhibited proliferation induced by PDGF-BB and serum (Figure 6A and 6B). The inhibitory effects of cicaprost on cell proliferation were attenuated by the addition of 10^{-2} M 2',5'-DDA (Figure 6A and 6C). Concomitant with the inhibition of DNA synthesis, cicaprost increased intracellular cAMP production in a concentration-dependent manner, and IBMX (5×10^{-5} M) potentiated the response, increasing cAMP production 2-fold (Figure 7A). Even without IBMX, cicaprost (10^{-7} M) induced a 4-fold increase in cAMP production in distal PASMCs maintained at 37°C, a plateau being reached within 15 to 30 minutes and maintained for 2 to 3 hours (Figure 7B). Indirect and direct stimulation of cAMP production, by cicaprost and forskolin, respectively, was partially inhibited by pretreatment with 10^{-4} M 2',5'-DDA. Submaximal stimulation (10^{-7} M cicaprost)
of cAMP production was reduced by \( \approx 50\% \) (19.97 ± 1.92 versus 10.98 ± 0.89 pmol/10^4 cells per well, \( P = 0.0053; n = 4 \)) in the presence of 2',5'-DDA but was unaffected by the addition of 10^{-6} to 10^{-4} mol/L SQ-22536 (data not shown).

The effects of cicaprost on cAMP production and the growth of distal PASMC isolates did not vary with the disease, age, or sex of the patients from which the cells were obtained. Regional differences in growth and responsiveness to adenylyl cyclase stimulation were observed in PASMCs derived from the same as well as different patients.

**Discussion**

There are 2 main findings in the present study. First, at least 2 SMC phenotypes were isolated from the human pulmonary artery bed; these differed in their proliferation rate and responsiveness to adenylyl cyclase stimulation. Second, PGI_2 analogues inhibited DNA synthesis and cell proliferation and concomitantly stimulated intracellular cAMP production in distal PASMCs. These effects were potentiated by IBMX and were mediated, at least in part, by a cAMP-dependent mechanism.

Heterogeneity in vascular SMCs is well recognized, with variations in cell phenotype and function occurring both within and between regions of systemic and pulmonary arteries. Several SMC phenotypes have been isolated from the main bovine pulmonary artery, the proportions and proliferation of which vary during development and in response to hypoxia. Distal human PASMCs exhibited growth characteristics similar to both adult bovine PASMCs, derived from the middle layer of the media, and human systemic arterial SMCs, whereas proximal PASMCs displayed a comparatively slow growth rate and were relatively unresponsive to adenylyl cyclase stimulation.

Vasodilatory prostaglandins have been found to promote rather than inhibit bovine PASMC proliferation, and the elevation of cAMP increased DNA synthesis in neonatal cells while having no apparent effect in adult bovine PASMCs. Conversely, we demonstrated consistent inhibition of DNA synthesis and proliferation after direct and indirect stimulation of intracellular cAMP production in human PASMCs. Bovine vascular SMCs are also reported to develop tolerance to the antimitogenic actions of iloprost over 24 hours. Human PASMCs, in contrast, exhibited a significantly greater inhibition of DNA synthesis when cicaprost was present for most of the 24-hour incubation period. These differences suggest regional heterogeneity among human PASMCs and possible species variations in their response to prostanoids. The regional heterogeneity may have important functional implications because vascular remodeling in the hypertensive pulmonary circulation is mainly a feature of distal resistance vessels rather than proximal arteries.

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A specific prostanoid receptor subtype, the IP receptor, mediates the actions of PGI_2 and is highly expressed in the human lung. The concentration-dependent effects of cicaprost on DNA synthesis and intracellular cAMP production in distal PASMCs correspond with the reported affinity of the IP receptor in ligand-binding studies. Although cicaprost and iloprost differ in their receptor selectivity, they exhibit comparable inhibitory effects on DNA synthesis in human PASMCs and possess a similar affinity for the IP receptor. Moreover, recent studies have demonstrated that both agonists cause relaxation of pulmonary artery preparations, with the IP receptor mediating prostanoid-induced relaxation of human pulmonary artery smooth muscle. Therefore, it seems likely that IP receptors mediate the actions of cicaprost and iloprost on DNA synthesis and proliferation of human PASMCs. Variation in receptor expression may contribute to regional differences in the inhibitory effects of PGI_2 analogues. However, direct adenylyl cyclase stimulation with forskolin also differentially inhibited DNA synthesis in distal versus proximal cells, suggesting variation in the expression or relative abundance of adenylyl cyclase and phosphodiesterase isoforms or counterregulatory mechanisms.
Several findings point to the involvement of the cAMP pathway in mediating the inhibitory effects of the PGI₂ analogues on human PASMCs. First, stimulation with isoproterenol, forskolin, or dbcAMP mimicked the inhibitory effect of the PGI₂ analogues on DNA synthesis in PASMCs. Second, the concentration-dependent effect of cicaprost on DNA synthesis corresponded to the stimulation of intracellular cAMP production. Furthermore, IBMX exerted a similar, albeit weaker, effect when added to PASMCs and potentiated the concomitant effects of cicaprost on DNA synthesis and cAMP production. Additional studies will be required to determine which phosphodiesterase enzymes are involved, with several isoforms having been identified in proximal human pulmonary arteries.29 Third, the inhibitory effect of cicaprost on PASMC proliferation occurred during progression from the G₀/G₁ to S phase of the cell cycle, concurring with previous studies demonstrating that the mitogenic activity of aortic SMCs was also inhibited at an early stage of the cell cycle.30 Last, 2’,5’-DDA attenuated both the acute stimulation of intracellular cAMP production and the inhibition of cell proliferation induced by cicaprost. The apparent ineffectiveness of SQ-22536 may reflect species and isoenzyme variations in either the specificity or sensitivity of adenylyl cyclase inhibitors.31 However, we cannot exclude the possibility that additional mechanisms are involved because the IP receptor can also activate other signaling pathways, including stimulation of inositol 1,4,5-triphosphate production, changes in intracellular Ca²⁺, and activation of K⁺ channels.32

Our findings indicate that there is regional heterogeneity among SMCs in human pulmonary arteries, with PGI₂ analogues selectively inhibiting the DNA synthesis and proliferation of distal PASMCs, at least in part, by stimulating intracellular cAMP production. The inhibition of PASMC growth may contribute to the response of patients with severe pulmonary hypertension to chronic treatment with PGI₂ and its analogues. It is possible that combined treatment with specific phosphodiesterase inhibitors and PGI₂ analogues will reduce PASMC proliferation further and thereby improve the hemodynamics and survival of these patients.
Acknowledgments
This study was supported by British Heart Foundation project grants PG96121 and PG98153. Dr. Morrell is a Medical Research Council Clinician Scientist Fellow.

References
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Circulation. 2000;102:3130-3136
doi: 10.1161/01.CIR.102.25.3130

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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