Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2010 September ; 93(1-2): 20–24. doi:10.1016/j.prostaglandins.2010.04.004.

Cell type- and cell cycle-specific anti-mitogenesis by cicaprost

Paola Castagnino, Devashish Kothapalli, Elizabeth A. Hawthorne, Tina Xu, and Richard K. Assoian

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6084

Keywords

prostacyclin; PGI₂; rapamycin; p27^{kip1}; vascular injury; endothelial cells; smooth muscle cells

1. Introduction

Drug-eluting stents (DES) have been increasingly used in an effort to reduce vascular smooth muscle cell (VSMC) proliferation after percutaneous coronary intervention, and stents eluting taxol or rapamycin have been widely used in this regard. However, recent work has indicated that these DES may themselves be complicated by an increased risk of thrombosis [1,2]. A current notion is that the risk of thrombosis results, at least in part, from the anti-proliferative effects of these agents on intimal cells, thereby impairing re-endothelialization after angioplasty.

Balloon- or wire-induced injury models demonstrate that VSMC proliferation plays a major role in neointima formation after vascular injury, and anti-proliferative therapies prevent the redevelopment of lesions in animal models [3–6]. Many ligands, receptors, and effector pathways regulate intimal hyperplasia after injury, but ultimately, all of these positive and negative regulators must converge on the cyclin-cdks, the enzymes responsible for mediating progression through the cell cycle. Rapamycin received attention as an anti-restenotic agent, at least in part, because of its ability to up-regulate levels of p27^{kip1} [7–9], a widely expressed protein that inhibits cyclin-dependent kinase (cdk)2 complexes in G1 and S phases [10]. However, rapamycin has been reported to inhibit endothelial cell (EC) proliferation [11]. The identification of anti-mitogenic agents selective for VSMCs would represent a significant advance in the treatment of restenosis.

We previously reported that the PGI₂ mimetic, cicaprost, also inhibits S phase entry by up-regulating p27^{kip1} levels [12,13]. PGI₂ is the major Cox2 product released by macrovascular ECs [14]. It is a potent vasodilator and inhibitor of platelet aggregation [15]. Deletion of the major PGI₂ receptor, IP [16,17], in mice results in an increased proliferative response after wire-induced injury [15], an effect that is likely due, at least in part, to its anti-mitogenic effect on VSMCs [12,13]. Studies by Li et al. revealed an inverse correlation between the effect of beraprost (a distinct PGI₂ mimetic) on p27^{kip1} levels and intimal hyperplasia in canines [18]. In this report, we show that cicaprost and rapamycin are both potent inhibitors of VSMC

© 2010 Elsevier Inc. All rights reserved.

Correspondence to: Richard K. Assoian, Ph.D., Department of Pharmacology, University of Pennsylvania School of Medicine, 3620 Hamilton Walk, Philadelphia, PA 19104-6084, Tel: 215-898-7157, Fax: 215-573-5656, assoian@mail.med.upenn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

proliferation. Rapamycin also inhibited cycling of aortic ECs whereas the anti-mitogenic effect of cicaprost was specific for VSMCs. Additionally, the anti-mitogenic effect of cicaprost on VSMC cycling is fully dependent on p27^{kip1} whereas the effect of rapamycin extends beyond p27^{kip1}. Our data indicate that stable PGI₂ mimetics may be highly specific inhibitors of p27^{kip1}-dependent VSMC proliferation after vascular injury.

2. Materials and methods

2.1 Cell culture and subcellular analysis

Mouse VSMCs were isolated from aortic explants of 10–12 week male wild-type (Taconic or Jackson Laboratories) or p27^{kip1}-null mice (Jackson Laboratories) on the C57BL/6 background. The cells were maintained in 10% FBS-DMEM-Ham's F12 and used to passage 5. Primary human aortic VSMCs were purchased from Cascade Biologics, maintained in 10% FBS-DMEM and used to passage 10. Human aortic ECs were purchased from Lonza and maintained in EBM-2 medium (Endothelial Basal Medium, Lonza) containing 2% FBS and a proprietary mix of hydrocortisone, hFGF-B, VEGF, IGF, ascorbic acid, hEGF, and heparin and used to passage 10. Near confluent VSMCs were serum-starved into quiescence by 2–3 day incubation in DMEM-1 mg/ml fatty acid-free, heat-inactivated BSA (1 mg/ml). The starved cells were directly stimulated with fresh maintenance medium. Near confluent aortic ECs were starved in EBM-2 medium, 1 mg/ml BSA for 24 h and directly stimulated with fresh maintenance medium. In most experiments, the cells were also treated with vehicle, rapamycin (Calbiochem) or cicaprost (generous gift of Bayer Schering Pharma). To analyze S phase entry, cells were seeded in dishes containing coverslips, incubated with BrdU, and analyzed by immunofluorescence microscopy [19]. BrdU incorporation for the FBS-treated WT and p27-null VSMCs cultured in the absence of rapamycin or cicaprost typically ranged from 40–60%. The incubation times with FBS we used for each cell type optimized cycling through G1 and S phase.

2.2 Western blotting and quantitative real-time RT-PCR (RT-qPCR)

Cells were washed twice with cold PBS, scraped, collected by centrifugation and then lysed in 50 µl of TNE buffer (50mM Tris-HCl, pH 8.0, 250 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 mM NaF and 10 mM Na₃VO₄). Protein concentration was determined by Coomassie binding (Bio-Rad Laboratories, Inc.; 500-0006) following the manufacturer's instructions. Equal amounts of protein from each lysate were loaded on reducing 10% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes and immunoblotted using standard procedures. Primary antibodies included anti-p27 (BD Transduction laboratories; 610241), anti-p21 (Santa Cruz, SC-6246), anti-p70 S6 kinase (kind gift of John Blenis), or anti-pT389-p70 S6 kinase (Cell Signaling; 9205). Filters were also probed with anti-GAPDH (Santa Cruz, SC-25778) or anti-actin (Santa Cruz, SC-8432) as loading controls. The fractionated proteins were visualized using enhanced chemiluminescence (Amersham ECL Western Blotting detection reagents; RPN2106). Films were digitized by scanning and the images were assembled in Photoshop (Adobe). Western blot results shown here are representative of multiple independent experiments (refer to Suppl. Tables 1 and 2). Densitometric analysis of the different experiments was performed using ImageJ software. RT-qPCR was performed as described [19] using SYBR green and 900 nM primers to human IP (Forward: ACACGAGGAGCAAAGCAAGT and Reverse: GCACGTAGGTGAGGTTCTG) and a TaqMan primer-probe set for 18S rRNA [19].

3. Results and Discussion

3.1 Increased cell cycle specificity of cicaprost vs. rapamycin

Our initial experiments compared the effects of rapamycin and cicaprost on cycling of primary aortic VSMCs. Rapamycin treatment of primary wild-type mouse VSMCs resulted in a dose-dependent increase in p27^{kip1} levels (Fig. 1A and Suppl. Table 1), and this effect correlated well with the ability of rapamycin to inhibit phosphorylation of p70 S6 kinase (Fig. 1A, an accepted read-out of rapamycin action through its inhibitory effect on mTORC1 [20]). Increased p27^{kip1} protein persisted from 24 h to 48 h in rapamycin-treated cells (Suppl. Fig. 1). Curiously, levels of the closely related cdk inhibitor, p21^{cip1}, were down regulated by rapamycin (Fig. 1A and Suppl. Fig 1).

The increased expression of p27^{kip1} correlated with a decrease in mitogenesis as determined by BrdU incorporation (Fig. 1B; closed circles). Like studies in embryonic fibroblasts [21], the anti-mitogenic effect of rapamycin persisted in VSMCs isolated from p27^{kip1}-null mice (Fig. 1B; closed squares) though the effect was less pronounced than in the wild-type cells. We also incubated rapamycin with wild-type or p27^{kip1}-null mouse embryo fibroblasts (Suppl. Fig. 2): the wild-type cells showed the expected time-dependent down regulation of p27^{kip1} and transient induction of p21^{cip1} after serum stimulation. Rapamycin blocked p27^{kip1} downregulation in a dose-dependent manner. However, S phase entry in both wild-type and p27^{kip1}-null MEFs was inhibited by rapamycin, and the relative degrees of inhibition were similar to those seen in the wild-type and p27^{kip1}-null VSMCs, respectively (refer to Fig. 1B). Thus, the anti-mitogenic effect of rapamycin in these mouse cells extends beyond regulation of p27^{kip1}.

When we added the PGI₂ mimetic, cicaprost, to early passage mouse VSMCs, we again obtained a strong upregulation of p27^{kip1} (Fig. 2A and Suppl. Table 2; 200 nM cicaprost). p21^{cip1} levels were much less affected by cicaprost (Fig. 2A). Notably, the concentrations of cicaprost that inhibited FBS-induced p27^{kip1} downregulation also inhibited DNA synthesis (Fig. 2B). Moreover, the anti-mitogenic effect of cicaprost was completely absent in p27^{kip1}-null mouse VSMCs (Fig. 2B). Thus, while the ultimate efficacy of rapamycin and cicaprost are similar in terms of cell cycle inhibition, the molecular specificity of cicaprost exceeds that of rapamycin.

We extended these studies to early passage human VSMCs and found that the effect of rapamycin on p27^{kip1} levels was barely detectable (Fig. 3A and Suppl. Table 1). Yet p70 S6 kinase phosphorylation and S phase entry were still inhibited by rapamycin (Fig. 3A and B). We note that the BrdU dose response curve for rapamycin in human VSMCs resembles the dose response curve for rapamycin in mouse VSMCs lacking p27^{kip1} (refer to Fig. 1B). This observation, and the lack of a strong rapamycin effect on p27^{kip1} levels support the conclusion that rapamycin has p27^{kip1}-independent effects on mitogenesis in VSMCs. In contrast, the inhibitory effects of cicaprost on p27^{kip1} downregulation (Fig. 4A and Suppl. Table 2) and S phase entry (Fig. 4B) was retained in human VSMCs.

3.2 Increased cell type-specificity of cicaprost versus rapamycin

Re-endothelialization of damaged blood vessels is a critical component of the response to injury, at least in part, because it allows for the establishment of a non-thrombogenic intima. Rapamycin, however, has been reported to inhibit the proliferation of ECs and endothelial progenitors [11,22]. Indeed, we found that rapamycin inhibited phosphorylation of p70 S6 kinase (Fig. 5A) and mitogenesis (Fig. 5B) in human aortic ECs; this effect is likely p27^{kip1}-independent since we were unable to detect a rapamycin-dependent up-regulation of p27^{kip1} (Fig. 5A and Suppl. Table 1). In striking contrast, mitogenesis of human aortic ECs was

completely resistant to cicaprost, even when used at concentrations that were 10-fold higher than those which fully inhibit cycling of human VSMCs (Suppl. Table 2; also compare 200 nM cicaprost in Fig. 4B with 200–2000 nM cicaprost in Fig. 6A). Similarly, the G1 phase levels of p27^{kip1} were unaffected by cicaprost in human aortic ECs (Fig. 6B and Suppl. Table 2).

PGI₂ signaling in ECs is regulated downstream of receptor gene expression

To begin to understand the basis for cell type-specific inhibitory effect of cicaprost, we asked if the receptor used to regulate p27^{kip1} might be differentially expressed in VSMCs and ECs. The major receptor for PGI₂ is IP [16,17], and indeed we found that cicaprost lost its ability to regulate p27^{kip1} levels in VSMCs isolated from IP-null mice (Fig. 7A). We then used RT-qPCR to determine if the IP gene was expressed in ECs as well as VSMCs. As shown in Fig. 7B, IP mRNA was detected in both cell types with a relatively small difference (<2-fold) in the level of expression. Moreover, Bunday et al. have reported that PGI₂ mimetics can regulate the permeability of EC monolayers if adenylyl cyclase 6 is overexpressed [23], indicating that IP protein is also expressed in these cells. The small difference in IP gene expression notwithstanding, our data and those of Bunday et al. collectively suggest that the major defect in PGI₂/IP-dependent cell cycle control in ECs is imposed downstream of IP expression.

Increased proliferation of VSMCs is accepted as a major determinant of the response to vascular injury. The VSMC proliferative response is thought to occur, at least in part, because platelets aggregate on intimal surfaces where reendothelization of the denuded intimal cells is incomplete. The data reported here indicate that the PGI₂ mimetic, cicaprost, has the important property of strongly inhibiting mitogenesis of arterial VSMCs but not arterial ECs. The cell type-specific effect of cicaprost on mitogenesis is markedly greater than that of rapamycin or a distinct PGI₂ mimetic, U-61,431F, reported in very early studies [24]. It also appears to exceed that of cerivastatin as described by Shirotani et al. [25]. Our studies with p27^{kip1}-null VSMCs indicate that p27^{kip1} likely represents the major cell cycle target of cicaprost, a finding consistent with our work showing that cicaprost does not affect the G1 phase levels of p21^{cip1} (a closely related cdk inhibitor) or cyclin D1 [12,13]. Collectively, these data indicate that the PGI₂ mimetic, cicaprost, is a highly specific inhibitor of p27^{kip1}-dependent VSMC proliferation. It is tempting to consider that these properties might be exploited pharmacologically to combat restenosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Bayer Schering Pharma for providing cicaprost. This work was supported by NIH grants HL083367 and HL62250.

References

1. McFadden EP, Stabile E, Regar E, Cheneau E, Ong AT, Kinnaird T, Suddath WO, Weissman NJ, Torguson R, Kent KM, Pichard AD, Satler LF, Waksman R, Serruys PW. Late thrombosis in drug-eluting coronary stents after discontinuation of antiplatelet therapy. *Lancet* 2004;364:1519–1521. [PubMed: 15500897]
2. Pfisterer M, Brunner-La Rocca HP, Buser PT, Rickenbacher P, Hunziker P, Mueller C, Jeger R, Bader F, Osswald S, Kaiser C. Late Clinical Events After Clopidogrel Discontinuation May Limit the Benefit of Drug-Eluting Stents: An Observational Study of Drug-Eluting Versus Bare-Metal Stents. *Journal of the American College of Cardiology* 2006;48:2584–2591. [PubMed: 17174201]

3. Yang Z, Simari RD, Tanner F, Stephan D, Nabel GJ, Nabel EG. Gene transfer approaches to the regulation of vascular cell proliferation. *Semin Interv Cardiol* 1996;1:181–184. [PubMed: 9552509]
4. Suzuki J, Isobe M, Morishita R, Aoki M, Horie S, Okubo Y, Kaneda Y, Sawa Y, Matsuda H, Ogihara T, Sekiguchi M. Prevention of graft coronary arteriosclerosis by antisense cdk2 kinase oligonucleotide. *Nat Med* 1997;3:900–903. [PubMed: 9256282]
5. MacLellan WR, Majesky MW. Cell cycle regulators in vascular disease. *Circulation* 1997;96:1717–1719. [PubMed: 9323050]
6. Chang MW, Barr E, Seltzer J, Jiang YQ, Nabel GJ, Nabel EG, Parmacek MS, Leiden JM. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. *Science* 1995;267:518–522. [PubMed: 7824950]
7. Marks AR. Sirolimus for the prevention of in-stent restenosis in a coronary artery. *N Engl J Med* 2003;349:1307–1309. [PubMed: 14523135]
8. Marx SO, Marks AR. Bench to bedside: the development of rapamycin and its application to stent restenosis. *Circulation* 2001;104:852–855. [PubMed: 11514367]
9. Woods TC, Marks AR. Drug-eluting stents. *Annu Rev Med* 2004;55:169–178. [PubMed: 14746515]
10. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1 phase progression. *Genes Dev* 1999;13:1501–1512. [PubMed: 10385618]
11. Matter CM, Rozenberg I, Jaschko A, Greutert H, Kurz DJ, Wnendt S, Kuttler B, Joch H, Grunenfelder J, Zund G, Tanner FC, Luscher TF. Effects of tacrolimus or sirolimus on proliferation of vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* 2006;48:286–292. [PubMed: 17204907]
12. Kothapalli D, Stewart SA, Smyth EM, Azonobi I, Pure E, Assoian RK. Prostacyclin receptor activation inhibits proliferation of aortic smooth muscle cells by regulating cAMP response element-binding protein- and pocket protein-dependent cyclin a gene expression. *Mol Pharmacol* 2003;64:249–258. [PubMed: 12869629]
13. Stewart SA, Kothapalli D, Yung Y, Assoian RK. Antimitogenesis linked to regulation of Skp2 gene expression. *J Biol Chem* 2004;279:29109–29113. [PubMed: 15126498]
14. Fetalvero KM, Martin KA, Hwa J. Cardioprotective prostacyclin signaling in vascular smooth muscle. *Prostaglandins Other Lipid Mediat* 2007;82:109–118. [PubMed: 17164138]
15. Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, FitzGerald GA. Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science* 2002;296:539–541. [PubMed: 11964481]
16. Coleman RA, Grix SP, Head SA, Louttit JB, Mallett A, Sheldrick RL. A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins* 1994;47:151–168. [PubMed: 8016385]
17. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev* 1999;79:1193–1226. [PubMed: 10508233]
18. Li M, Hoshiga M, Fukui R, Negoro N, Nakakoji T, Nishiguchi F, Kohbayashi E, Ishihara T, Hanafusa T. Beraprost sodium regulates cell cycle in vascular smooth muscle cells through cAMP signaling by preventing down-regulation of p27(Kip1). *Cardiovasc Res* 2001;52:500–508. [PubMed: 11738067]
19. Klein EA, Yung Y, Castagnino P, Kothapalli D, Assoian RK. Cell adhesion, cellular tension, and cell cycle control. *Methods Enzymol* 2007;426:155–175. [PubMed: 17697884]
20. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. 2009;10:307–318.
21. Luo Y, Marx SO, Kiyokawa H, Koff A, Massague J, Marks AR. Rapamycin resistance tied to defective regulation of p27Kip1. *Mol Cell Biol* 1996;16:6744–6751. [PubMed: 8943329]
22. Imanishi T, Kobayashi K, Kuki S, Takahashi C, Akasaka T. Sirolimus accelerates senescence of endothelial progenitor cells through telomerase inactivation. *Atherosclerosis* 2006;189:288–296. [PubMed: 16455087]
23. Bunday RA, Insel PA. Adenylyl cyclase 6 overexpression decreases the permeability of endothelial monolayers via preferential enhancement of prostacyclin receptor function. *Mol Pharmacol* 2006;70:1700–1707. [PubMed: 16885208]

24. Shirotani M, Yui Y, Hattori R, Kawai C. U-61,431F, a stable prostacyclin analogue, inhibits the proliferation of bovine vascular smooth muscle cells with little antiproliferative effect on endothelial cells. *Prostaglandins* 1991;41:97–110. [PubMed: 1708156]
25. Jaschke B, Michaelis C, Milz S, Vogeser M, Mund T, Hengst L, Kastrati A, Schomig A, Wessely R. Local statin therapy differentially interferes with smooth muscle and endothelial cell proliferation and reduces neointima on a drug-eluting stent platform. *Cardiovasc Res* 2005;68:483–492. [PubMed: 16111664]

Fig. 1. Anti-mitogenic effects of rapamycin extend beyond p27^{kip1} in mouse VSMCs

Serum-starved mouse VSMCs isolated from WT and p27-null mice were directly stimulated with fresh 10% FBS-DMEM and increasing concentrations of rapamycin for 48 h. **(A)** Cells were collected, lysed and analyzed by western blotting. **(B)** Coverslips were fixed and analyzed for BrdU incorporation. BrdU results show mean $\pm$ SD (n=3).

Fig. 2. The anti-mitogenic effect of cicaprost requires p27^{kip1} in mouse VSMCs

Serum-starved mouse VSMCs isolated from WT and p27-null mice were directly stimulated with fresh 10% FBS-DMEM and increasing concentrations cicaprost. **(A)** Western blot analysis was performed after 24 h. **(B)** WT and p27-null VSMCs were stimulated with 10% FBS-DMEM and increasing concentrations cicaprost for 48 h before analysis of S phase entry by BrdU incorporation. Results in B show the mean $\pm$ SD (n=3).

Fig. 3. Anti-mitogenic effects of rapamycin in human VSMCs

Serum-starved human VSMCs were directly stimulated with 10% FBS and increasing concentrations of rapamycin. **(A)** Cells were collected and lysed after 24 h for analysis by western blotting. **(B)** Coverslips were fixed after 48 h and analyzed for BrdU incorporation. BrdU results show the mean $\pm$ SD (n=3).

Fig. 4. Anti-mitogenic effect of cicaprost in human VSMCs

The experiments in Fig. 3A and B, respectively, were repeated using cells treated with increasing concentrations of cicaprost rather than rapamycin. BrdU results show mean $\pm$ SD (n=3).

Fig. 5. Rapamycin inhibits mitogenesis of aortic endothelial cells

Serum-starved human aortic ECs were directly stimulated with mitogens in the presence of BrdU and increasing concentrations of rapamycin for 24 h. **(A)** Cells were collected, lysed, and analyzed by western blotting. **(B)** Coverslips were fixed and analyzed for incorporation of BrdU. BrdU results show mean $\pm$ SD (n=4).

Fig. 6. Aortic endothelial cells are resistant to the anti-mitogenic effect of cicaprost
The experiments in Fig. 5A and B were repeated in the presence of increasing concentrations of cicaprost rather than rapamycin. BrdU results show mean $\pm$ SD (n=4).

Fig. 7. The defect in PGI₂ signaling to p27^{kip1} in endothelial cells is imposed downstream of IP gene expression

(A) Mouse VSMCs isolated from wild-type or IP-null mice were serum-starved and then directly stimulated with 10% FBS in the absence or presence of 200 nM cicaprost. Cells were collected after 24 h and analyzed by western blotting for p27^{kip1} and actin. **(B)** Asynchronous human VSMCs (HSMC) and aortic ECs (HAEC) were analyzed by RT-qPCR for the levels of IP mRNA and 18S rRNA. IP gene expression is plotted relative to 18S rRNA. Results show mean $\pm$ SD of duplicate PCR reactions.