Beraprost sodium regulates cell cycle in vascular smooth muscle cells through cAMP signaling by preventing down-regulation of p27^Kip1

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Abstract

**Objective:** Beraprost sodium (BPS), a prostacyclin (PGI2) analogue, has been reported to exhibit beneficial effects on atherosclerosis in both human and animal models. To clarify the underlying mechanism, we investigated the effects of BPS on neointimal formation after balloon injury in the canine coronary artery. Furthermore, we determined its anti-atherosclerotic effects in cultured smooth muscle cells (SMCs).

**Methods:** Adult beagle dogs (10–12 kg) were fed on a high-cholesterol diet (10 g/day) and underwent balloon-denudation of the coronary artery. The dogs were divided into two groups: a BPS-treated group (20 mg/kg per day) and a control group. Twenty-eight days after injury, the dogs were killed and the coronary arteries were examined morphometrically. Three days after injury, the proliferative activity in the medial layer of the coronary artery was evaluated by 5-bromo-2-deoxyuridine (BrdU) incorporation, and Kip1, a cyclin-dependent kinase (cdk) inhibitor, expression was examined by immunohistochemistry. We also examined the effects of BPS on SMC proliferation based on BrdU incorporation and cell cycle analysis. In addition, p27^Kip1 regulation was evaluated in primary-cultured SMCs.

**Results:** BPS administration decreased the intima/media ratio (I/M) by 88% in the control group. Three days after injury, BPS attenuated the proliferation rate of the cells in the media of the coronary artery by 35%, and maintained p27^Kip1 expression, which declined in the control cells. In the cultured proliferating SMC, BPS prevented the down-regulation of p27^Kip1. The 8-bromo-cyclic adenosine monophosphate (8-br-cAMP), a cAMP analogue, had similar actions as BPS in the regulation of p27^Kip1. The proliferation of cultured SMC was inhibited in a dose-dependent manner, and cell cycle arrest in the G1 phase was induced by BPS.

**Conclusions:** Our data suggest that BPS inhibits neointimal formation after balloon denudation in the coronary artery through its inhibitory effect on SMC proliferation by preventing p27^Kip1 down-regulation. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Angioplasty; Protein kinases; Prostaglandins; Restenosis; Smooth muscle

1. Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is a widely used therapy for coronary artery disease. However, its efficacy is limited by the restenosis that occurs in one-third of the patients within 6 months after the procedure [1,2]. Although stent development reduced the restenosis rate after coronary intervention [3,4], in-stent restenosis still occurs in about 20% of the patients after the procedure, giving rise to a new and significant clinical problem. It is well documented that the neointima formed after arterial stenting is composed mainly of vascular smooth muscle cell (VSMC) and its proliferation plays an important role in the restenotic process [5,6]. Therefore, investigation into VSMC proliferation inhibition after arterial injury is important. It is also beneficial for the clinical therapy of spontaneous atherosclerosis and post-transplantation atherosclerosis as well as for restenosis after angioplasty.

The molecular mechanisms responsible for VSMC proliferation have been intensively studied. One of particular note is the Ras pathway [7] and cyclic-3',5'-adenosine monophosphate-protein kinase A (cAMP-PKA) intracellular signaling, because activation of cAMP-PKA signaling...
inhibits VSMC proliferation in vitro [8], making it a good candidate for preventing vascular proliferative disease. It has very recently been reported that 8-chloro (Cl)-cAMP, a cAMP analogue, inhibits VSMC proliferation in vitro and reduces neointimal formation in the rat carotid artery by balloon injury [9]. However, there are clinical limitations to designing future strategies using 8-Cl-cAMP to prevent restenosis after PTCA, particularly in arterial stenting, because this agent has been investigated as an anticancer agent and has some side effects in humans [10]. We therefore focused on cAMP-elevating agents other than cAMP analogues, such as prostacyclin (PGI₂) or a phosphodiesterase inhibitor. Beraprost sodium (BPS) is a stable analogue of PGI₂ that increases intracellular cAMP levels via activation of adenylate cyclase [11]. BPS has been used for peripheral vascular disease without major side effects. One clinical trial showed that administration of BPS reduces restenosis after PTCA [12]. In animal studies, BPS has been reported to reduce intimal hyperplasia in rat [13] and rabbit [14]; however, the mechanism responsible for its inhibitory effects on the intimal hyperplasia is unknown. The purpose of this study is to determine the effect of BPS in coronary injury model, and to elucidate its molecular mechanism, in particular, cell cycle regulation in VSMC.

2. Methods

2.1. Animal study

The animal study conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult female beagle dogs (10–12 kg) were fed on a high cholesterol diet (10 g/day) for 3 months, and were randomly assigned to two groups: a test group that received BPS (Kaken Pharmaceutical, Tokyo, Japan) for 7 days before injury (20 μg/kg per day, orally, n=8) and a control group that did not receive BPS (n=8). The animals were anesthetized with pentobarbital (30 mg/kg i.v.) and then intubated. A PTCA balloon (balloon:artery ratio=1.2) was inserted into the left circumflex artery (LCX) via the right common carotid artery under fluoroscopic guidance and denuded twice by inflating the balloon to 4 atm. After the procedure, all devices were removed and the puncture site was closed with suture. Twenty-eight days following the procedure, all devices were removed and the puncture computer software (NIH image right common carotid artery under fluoroscopic guidance chemiluminescent detection method of ECL (Amersham). was inserted into the left circumflex artery (LCX) via the peroxidase-conjugated anti-mouse antibody and the then intubated. A PTCA balloon (balloon:artery ratio were anesthetized with pentobarbital (30 mg / kg i.v.) and in 0.05% Tween 20±phosphate-buffered saline (PBS). (Nikon, Tokyo, Japan). 5% CO in air. All cells (1×10⁶ cells, between passages 3

2.2. Immunohistochemistry

Three days after balloon injury, BrdU (500 mg/body) was administered intramuscularly to the dogs (the control group n=3 and the BPS group n=3) 10 h before sacrifice. Specimens of the LCX were prepared in the same way as described above. After deparaffinization and hydration, endogenous peroxidase was blocked by immersion in 3% H₂O₂ [15]. The specimens were then exposed to high-power microwaves twice for 10 min separated by a 5-min interval at 4°C. After adding serum-blocking solution (LSAB kit, Dako, Tokyo, Japan), the primary antibody (anti-BrdU antibody (1:500) or anti-p27kip antibody (1:1000), PharMingen) was incubated overnight at 4°C. After the samples were washed, a biotinylated secondary antibody was added, and color development was carried out according to the instructions of the manufacturer (LSAB kit, Dako). The slides were counterstained with 0.25% methyl green.

2.3. Western blot analysis

For the Western blot analysis, the endothelium and the adventitia were carefully removed from the coronary artery 3 days after injury. Sections of isolated coronary artery were homogenized using a tissue homogenizer and cells were scraped into lysis buffer (10% trichloroacetic acid (TCA)–3 mM dithiothreitol (DTT)) at 4°C. Extracts were centrifuged at 3000 rpm for 10 min, and the pellets were rinsed with ether and either used immediately or stored at −70°C. Protein concentration was measured by the Bradford method (Bio-Rad, San Diego, CA, USA). Extracts containing equal amounts of protein were denatured by boiling for 5 min in 50 mM Tris–HCl, pH 6.5, 5% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM DTT, and electrophoresed on 10% SDS–PAGE gels at 120 V. The protein bands were then transferred to a PVDF membrane (Amersham) at 10 V/cm for 1 h. The membrane was rinsed and blocked with 5% non-fat dry milk for 1 h and then incubated overnight at 4°C with primary antibody in 0.05% Tween 20–phosphate-buffered saline (PBS). Immune complexes were detected with a horseradish peroxidase-conjugated anti-mouse antibody and the chemiluminescent detection method of ECL (Amersham). Band intensities were measured using image analyzing computer software (NIH image™).

2.4. In vitro p27kip expression

SMCs were obtained from the canine coronary arteries by the modified explant method [16]. Briefly, we used 10 ng/ml of human endothelium growth factor (hEGF) (Life Technologies Oriental, Tokyo, Japan)/Eagle minimum essential medium (MEM) for maintenance of cultured cells instead of 10% fetal calf serum (FCS)/MEM at 37°C in 5% CO₂ in air. All cells (1×10⁶ cells, between passages 3
and 8) were maintained in hEGF-free medium for 48 h and preincubated for 30 min in MEM either with or without BPS (Toray Industries, Tokyo, Japan) at a concentration of 10 μmol/l. Samples were harvested at 0, 12, 18, and 24 h after stimulation by hEGF (10 ng/ml). In addition, cells were harvested at 18 h after proliferative stimulation in the presence or absence of BPS (10 μmol/l), 8-bromo-cyclic adenosine monophosphate (8-br-cAMP; 1 mmol/l), and 2′, 5′-dideoxyadenosine (DDA; 100 μmol/l). These samples were examined for the expression of p27Kip1 by Western blot as described above.

2.5. In vitro SMC proliferation assay

Cells were plated on Lab-Tek chamber slides (Nalge Nunc, Rochester, NY, USA) at a density of 1×10⁵ cells/well, and maintained in 10 ng/ml of hEGF/MEM with BPS (Toray) at concentrations of 0, 0.1, 1, or 10 μM after 48 h of hEGF deprivation. After 18 h, BrdU (5 μmol/l; Sigma, St. Louis, MO, USA) was added to each well. BrdU incorporation was examined by immunocytochemistry after 6 h as described previously [17]. BrdU-positive cell counts in three fields/well were averaged and used to assess proliferative activity.

2.6. Flow cytometry

Quiescent cells were stimulated by 10 ng/ml of hEGF in the presence or absence of BPS (10 μmol/l) (Toray), and harvested at 0, 12, 18, and 24 h after stimulation. After trypsinization, the cells were stained with acridine orange. The number of cells at different phases of the cell cycle was evaluated using a flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an argon ion laser (488 nm) at a flow rate of 1000–1200 cells/s [18]. The red (630 nm) and the green (525 nm) fluorescence emissions from each cell were separated optically and quantified by separate photomultipliers. The intensity of red and green fluorescence was proportional to the amount of RNA and DNA, respectively. The specificity of the detection of the DNA and RNA contents was evaluated by preincubating permeable cells with DNase I (1 mg/ml, Sigma) and RNase A (1 mg/ml, Sigma). At least 91% of both the red and green fluorescent signals were the result of acridine orange interaction with cellular RNA and DNA, respectively. Standardized beads were used as an external standard.

2.7. Statistical analysis

All values were reported as the mean±S.E.M. Statistical analysis was performed using commercially available statistical software (Statview™). When two groups were compared, differences were analyzed using the unpaired Student’s t-test. A value of P<0.05 was considered significant.

3. Results

3.1. Coronary injury model and the effect of BPS on serum lipid levels

A thick neointimal layer did not develop in the dogs fed on a normal diet, but did form in dogs fed on a high-cholesterol diet 28 days after balloon injury (Fig. 1A). Therefore, we used this model to examine the effects of BPS. The cholesterol feeding significantly increased the serum concentration of cholesterol (131.2±12.0 in the normal diet group, n=10, vs. 234.9±31.3 mg/dl in the cholesterol diet group, n=8, P<0.001). However, BPS treatment did not affect the serum concentration of cholesterol (234.9±31.3 in the cholesterol diet group, n=8, vs. 236±33.7 mg/dl in the cholesterol diet+BPS group, n=8, NS). The serum concentration of triglycerides did not change significantly between each groups (41.0±2.5 in the normal diet group, 40.4±3.8 in the cholesterol diet group and 44.2±14.4 mg/dl in the cholesterol diet+BPS group).

3.2. Inhibition of neointimal formation after injury

BPS treatment markedly inhibited the neointimal formation 28 days after balloon injury (Fig. 1A). Morphometric analysis revealed that BPS administration decreased the neointimal area by 92% (1.84±0.36 in the control group, n=5, vs. 0.15±0.01 mm² in the BPS group, n=5, P<0.001) and I/M by 88% (0.90±0.21 in the control group vs. 0.11±0.01 in the BPS group, P<0.001). In this model, the luminal area decreased in the control group 28 days after injury, while BPS inhibited this late loss in the luminal area (2.25±0.79 in the control group vs. 3.04±0.30 mm² in the BPS group, P<0.01). BPS had little effect on the medial area (2.06±0.07 in the control group vs. 1.88±0.05 mm² in the BPS group, NS).

3.3. Inhibitory effect of BPS on medial SMC proliferation after injury

To examine the effect of BPS on proliferative activity, BrdU labeling was determined 3 days after balloon injury. The percentage of BrdU incorporated in the media was calculated by dividing the number of BrdU-positive cells by the total number of cells (BrdU-positive cells plus BrdU-negative cells). The BrdU-negative cells appeared as light green nuclei stained with methyl green. The nuclei were counted under the microscope in any three fields and the data were averaged. The percentage of BrdU-positive cells in the BPS group was significantly lower than that in the control group (30.3±1.9 in the control group vs. 19.7±1.1% in the BPS group, P<0.005, Fig. 1B). These findings suggest that BPS inhibited SMC proliferation in the coronary artery after balloon injury.
Fig. 1. Inhibition of neointimal formation by BPS after balloon injury. Van Gieson-stained sections of coronary arteries 28 days after injury in control and BPS-treated animals (A) (original magnification, ×40). BPS inhibited SMC proliferation in the media after injury in vivo. BrdU staining 3 days after injury in control or BPS-treated animals (B). BrdU-positivity was significantly decreased by BPS. Arrows indicate BrdU-positive cells in the media (original magnification, ×200). Closed arrowheads indicate the internal elastic lamina (IEL); open arrowheads, external elastic lamina (EEL). M, media; I, intima; and L, lumen.

3.4. Effect of BPS on endogenous p27\textsuperscript{Kip1} expression in the media after injury

To investigate the mechanisms responsible for the inhibitory effect of BPS on cell proliferation in coronary arteries, endogenous expression of cell cycle inhibitors was determined. There was minimal expression of p21\textsuperscript{Cip1} and p16 in the canine coronary artery before and after injury (data not shown). In contrast, p27\textsuperscript{Kip1} expression was abundant in non-injured medial SMC, and it was strikingly decreased at 3 days after balloon injury (Fig. 2). BPS treatment inhibited the reduction in p27\textsuperscript{Kip1} expression after injury, as determined by immunohistochemistry (Fig. 2) and Western blot analysis (8.2±2.1 in the control group vs. 78.4±9.6% in the BPS group, P<0.01, Fig. 3).

3.5. Inhibition of SMC proliferation and G1 arrest by BPS

To study the effects of BPS on SMC proliferation, we used primary-cultured SMCs obtained from the canine coronary arteries between passages 3 and 8. BPS inhibited hEGF-induced BrdU incorporation in a dose-dependent manner with a maximum inhibition of 56% at a concentration of 10 \textmu mol/l (Fig. 4A). In addition, we examined the effect of BPS on the cell cycle phase using flow cytometry. Control cells were in the G1b (18±2%) or S (35.7±6.7%) phase at 18 h after hEGF stimulation. In contrast, the BPS-treated cells were in the G1b (60.4±1.6%) or the S (19.2±3.9%) phase at the same time point (Fig. 4B). The percentage of cells in the G1b phase
Fig. 2. Preventive effect of BPS on the decrease in endogenous p27<sub>Kip1</sub> expression in injured arteries. Immunohistochemistry using an antibody directed against p27<sub>Kip1</sub> in non-injured and injured arteries 3 days after injury in control and BPS-treated animals. Arrows indicate p27<sub>Kip1</sub>-positive cells in the media (original magnification, ×200). Closed arrowheads indicate the internal elastic lamina (IEL); open arrowheads, the external elastic lamina (EEL). M, media; I, intima; and L, lumen.

(18±2 in the control group vs. 60.4±1.6% in the BPS group, P<0.01) and the percentage of cells in the S phase were lower in BPS-treated cells. Similar results were also observed 24 h after stimulation.

3.6. p27<sub>Kip1</sub> expression of SMC in vitro

Expression of p27<sub>Kip1</sub> was dramatically decreased 18 and 24 h after hEGF stimulation in SMC according to the Western blot analysis (Fig. 5A). BPS treatment blocked the reduction of p27<sub>Kip1</sub> expression after hEGF stimulation. To elucidate the mechanism responsible for preventing the reduction in p27<sub>Kip1</sub> by BPS, we examined the effects of the cAMP analogue 8-br-cAMP (1 mmol/l) or co-incubation with BPS (10 μmol/l) and the adenylate cyclase inhibitor DDA (100 μmol/l) 18 h after stimulation. Treatment with 8-br-cAMP or BPS clearly inhibited the decrease in p27<sub>Kip1</sub> expression, whereas treatment with BPS plus DDA failed to inhibit this reduction (Fig. 5B). These findings suggest that BPS affects p27<sub>Kip1</sub> expression through cAMP signaling.

4. Discussion

The major findings of the present study are: (1) BPS, a stable PGI<sub>2</sub> analogue, inhibited the neointimal formation after balloon injury in the coronary artery; (2) BPS prevented SMC proliferation both in vivo and in vitro by, at least in part, maintaining endogenous expression of cdk inhibitor p27<sub>Kip1</sub>; and (3) the inhibitory effect of BPS on p27<sub>Kip1</sub> down-regulation appears to be through cAMP signaling.

BPS is developed as an oral stable PGI<sub>2</sub> and is already in clinical use for peripheral artery disease in Japan. PGI<sub>2</sub> has been reported to act as a vasorelaxant and anti-platelet aggregation via adenylate cyclase activation leading to an increase in intracellular cAMP concentrations [11]. Several lines of evidence suggest that cAMP suppresses vascular SMC proliferation by different mechanisms [8,19,20]: inhibition of the mitogen-activated protein (MAP) kinase cascade [21–23] and the cdk 4 pathway [24], which can ultimately inhibit cell progression. It has recently been
Fig. 4. Analysis of SMC proliferation and cell cycle phase. SMC proliferative activity was evaluated as the percentage of BrdU incorporation. BrdU positivity was measured 24 h after stimulation by hEGF (10 ng/ml) in the presence of varying BPS concentrations (A). The cell cycle phase for SMC was determined by flow cytometry 0, 12, 18, and 24 h after stimulation by hEGF (10 ng/ml) in the presence or absence of BPS (10 μmol/l) (B). *P<0.01 versus control.

shown that activation of the cAMP–protein kinase A (PKA) pathway inactivates Ras [21,25] and inhibits the M-phase-promoting factor (MPF) [26]. Ras has been shown to induce the G1–S transition and accelerate degradation of p27^kip1 through activation of the MAP kinase-signaling pathway. It has also been reported that cAMP-elevating agents prevent the Ras-induced degradation of p27^kip1 [27,28]. These findings support our data that BPS reduces neointimal formation in the injured artery via its effect on SMC proliferation. p27^kip1 is one of the cdk inhibitors that plays a major role in regulating cell cycle machinery [29–31], and has been shown to mediate cell cycle arrest in response to various factors, including the transforming growth factor (TGF)-β [32], rapamycin [33], cAMP [24], and the extracellular matrix [34]. The expression of p27^kip1 in the vascular wall may be a key to preventing VSMC proliferation, because balloon injury down-regulated p27^kip1 expression in SMCs [35] and
exogenous overexpression of $p27^{kip1}$ in the vessel wall successfully inhibits injury induction of SMC proliferation [36]. The degradation of $p27^{kip1}$ is known to be due to ubiquitin-dependent proteosomal degradation [37,38]. The reduction of $p27^{kip1}$ by balloon injury may be mediated by this signaling pathway in our model. Recently, it has been reported that $p27^{kip1}$ down-regulation in human vascular SMC [39] or cAMP-induced cellular morphological change in the human neuroblastoma cell line [40] is induced by the activated Rho GTPase. Therefore, to clarify the detail mechanism of the cAMP-signaling pathway for $p27^{kip1}$ degradation, further study is required on the relationship between cAMP and Rho.

In our canine coronary artery injury model, cholesterol feeding was necessary to cause prominent neointimal formation. Unlike in rabbits and pigs, cholesterol feeding does not produce fatty plaques in dogs; the developing neointima is mainly composed of dedifferentiated SMCs (data not shown). Therefore, the injured coronary artery in this model resembles the human coronary artery with early atherosclerosis or restenosis after PTCA [41]. In this model, the luminal area was significantly decreased 4 weeks after injury ($3.19\pm0.56$ in the non-injured arteries versus $2.40\pm0.34$ mm$^2$ in the injured arteries, $P<0.05$). Most studies using balloon injury in animals show no lumen loss in the vessel with neointima. This is probably due to adaptive remodeling, which was described earlier for the human coronary [42]. In our model, late lumen loss occurred, and BPS prevented both the lumen loss and neointimal formation. This may be due to vasorelaxant effect of BPS, and is thought to be an additional benefit for clinical use.

In conclusion, the present study indicates that BPS inhibits the neointimal formation after balloon injury in the canine coronary artery by its inhibitory effects on SMC proliferation. Its antiproliferative effect appears to be due
to maintaining p27^{Kip1}, even after proliferative stimuli, by BPS administration through cAMP signaling pathway. This inhibitory effect on p27^{Kip1} elimination is a novel action of BPS and is clinically applicable to various proliferative diseases, in particular, restenosis after PTCA.

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