Effects of Cilostazol, a Selective Cyclic AMP Phosphodiesterase Inhibitor on Isolated Rabbit Spinal Arterioles

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Abstract: Cilostazol, a potent inhibitor of guanosine 3':5'-cyclic monophosphate (cGMP)-inhibited adenosine 3':5'-cyclic monophosphate (cAMP) phosphodiesterase (PDE3), has been used clinically for the treatment of chronic peripheral arterial occlusive disease. The beneficial effect of cilostazol is attributed to both anti-platelet aggregating activity and vasodilation. However, the effect of cilostazol on resistance-sized vasculature is not well documented. Furthermore, mechanisms of vasodilation and influence on endothelium function are not fully understood. Thus, we investigated the vasodilator action of cilostazol using isolated, pressurized rabbit spinal arterioles with special reference to the functional endothelium. Cilostazol, acetylcholine (ACh), isocarbacyclin (prostacyclin analogue), and sodium nitroprusside (SNP) all produced concentration-dependent vasodilations of isolated spinal arterioles with endogenous myo-

genic tone. The order of potency of these agonists was isocarbacyclin>ACh>SNP>cilostazol. Indomethacin (10 μ M, a cyclo-oxygenase inhibitor), N^ω-nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthase inhibitor, 30 μ M), or chemical denudation of the endothelial cells did not significantly alter the cilostazol-induced arteriolar dilation. Furthermore, stimulating the release of endothelium-derived relaxing factors by administering ACh (100 nM), or treating with isocarbacyclin (1 nM) or SNP (3 nM) did not significantly modify the cilostazol-induced vasodilation. These results suggest that cilostazol produces the vasodilation of isolated, pressurized rabbit spinal arterioles independent of the functional endothelium. We infer that the vasodilator action of cilostazol in the spinal arterioles may be attributed to a yet unknown mechanism that is independent of the PDE3 inhibition. [Japanese Journal of Physiology, 52, 471–477, 2002]

Key words: spine, arteriole, cilostazol, vasodilation, endothelium.

Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone, OPC-13013) is an adenosine 3':5'-cyclic monophosphate (cAMP) phosphodiesterase (PDE) inhibitor known especially as a potent antithrombotic drug, which exerts a selective inhibition of guanosine 3':5'-cyclic monophosphate (cGMP)-inhibited cAMP phosphodiesterase (PDE3) in platelets [1–3]. Cilostazol has been used clinically for the treatment of chronic arterial occlusive disease [4]. Several clinical trials have shown that cilostazol significantly improves exercise performance in intermittent claudication, the most common symptom of lower-extremity peripheral artery occlusive

disease [5–8]. A recent clinical trial using a randomized, placebo-controlled, double-blind method also showed that long-term administration of cilostazol was safe and effective in preventing the recurrence of cerebral infarction, especially lacunar infarction [9]. The mechanism by which cilostazol benefits the cerebral circulation is not fully understood. However, the vasorelaxant effect of cilostazol, as well as the inhibition of platelet aggregation may, in part, contribute to its beneficial action. In support of this idea, cilostazol was reported to dilate pial arteries and inhibit the formation of thrombi during focal ischemia in the cat brain *in vivo* [10]. In humans, long-term oral adminis-

Received on March 14, 2002; accepted on September 17, 2002

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tration of cilostazol was demonstrated to increase cerebral blood flow in patients with chronic cerebral infarction [11]. Since lacunar infarction is defined as a small deep infarction that involves occlusion of small penetrating arterioles [12], it is of interest whether or not cilostazol elicits cerebral vasodilation at the arteriolar level. Plus, in spite of the numerous clinical and experimental findings on cilostazol, little information exists regarding the mechanisms by which cilostazol exerts vasodilation in arterioles.

Cilostazol elicits an anti-thrombotic effect via the inhibition of PDE3 and subsequent increase of cAMP in platelets. By the same logic, the vasodilator action of cilostazol is assumed to be due to PDE3 inhibition and corresponding cAMP increase in smooth muscles since PDE3 is strongly expressed in smooth muscle cells [13, 14]. Endothelium releases endogenous vasodilators such as nitric oxide (NO) and vasodilator prostaglandins in response to various chemical and mechanical stimuli. NO is well known to activate guanylate cyclase in vascular smooth muscles and increase cGMP in the cells, which may theoretically attenuate the PDE3 activity. Vasodilator prostaglandins, on the other hand, may potentiate the vasodilator action of cilostazol by increasing cAMP in the smooth muscles via activating adenylate cyclase [15–17]. Thus, although cilostazol and other PDE3 inhibitors are shown to exert vasodilation even in the absence of intact endothelium [10, 13, 18, 19], it is of particular interest whether or not endothelium or endothelium-derived factors can modulate the vasodilator action of cilostazol.

Our laboratory has been studying spinal circulation in dogs [20, 21] and rabbits [22], which could have significant physiological and clinical relevance as a regulator of the central nervous system. We found that isolated, pressurized arterioles from the rabbit spine are a highly suitable preparation for an *in vitro* analysis of arterioles because: (1) It is relatively easy to dissect as it possesses little connective tissues surrounding the vessel; (2) the distance between branches is substantially long (1–2 mm), which is perfect for cannulation at both sides without leakage; and (3) arterioles develop spontaneous myogenic tone (~60% of the passive diameter), which allows studies of vasodilator agents without any agonist-induced pre-constriction. The data obtained with spinal arterioles may be extrapolated to cerebral microcirculation as the same arteriole feeding the central nervous system. We have therefore attempted to examine the effects of cilostazol on pressurized arterioles (~110 μm passive diameter) isolated from rabbit spine with special reference to NO and vasodilator prostaglandins.

MATERIALS AND METHODS

Preparation and dissection. The Shinshu University School of Medicine Animal Ethics Committee approved the procedures and protocols used in this study. Nineteen Japanese white rabbits (1–3-month-old, 1.5–3.0 kg) were anesthetized with pentobarbital sodium (40 mg/kg, i.v.) and exsanguinated. Detailed methods for the dissection and cannulation of the spinal arterioles have been described previously [22]. Briefly, the spinal cord (lumbar portion) was rapidly removed and placed in a cooled (4°C) dissection chamber filled with 3-(N-morpholino) propane-sulfonic acid (MOPS)-buffered physiological salt solution containing 1% dialyzed bovine serum albumin (BSA). A spinal arteriole (~110 μm in passive diameter and ~3 mm long) was carefully dissected and transferred to a temperature-controlled cannulation chamber mounted on the stage of an inverted microscope (model IMT-2, Olympus, Tokyo, Japan). The arteriole was cannulated at both ends with a system of concentric glass pipettes (a perfusion pipette within a holding pipette) and connected to water manometers used to adjust intraluminal pressure via independent reservoirs (Fig. 1). After the arteriole was set to its *in situ* length, the intraluminal pressure was set at 60 mmHg [22]. Upstream and downstream reservoirs were set at the same height so that no flow is established in the lumen. The organ chamber was gradually heated to $37.0 \pm 0.5^\circ\text{C}$, and was suffused with MOPS solution without albumin at a constant flow rate (9 ml/min). The image of the pressurized arteriole was

Fig. 1. Experimental layout of cannulation system, tissue chamber, and apparatus for measurement of internal diameter of the isolated arterioles.

displayed on a television monitor, and luminal diameters were measured manually using a video caliper incorporated with MacLab Chart v3.2 (AD Instrument Inc., Castle Hill, Australia).

Experimental protocols. The arterioles were equilibrated for at least 60 min, during which time they developed a myogenic tone. After equilibration, three sets of experiments were performed. In the first series, changes in the diameter of arterioles were measured before and during the application of cilostazol, acetylcholine (ACh), isocarbacyclin (a stable prostacyclin analogue), or sodium nitroprusside (SNP), and dose-response curves for these agents were compared. These drugs were applied cumulatively into the organ chamber from the extraluminal side by 10-fold concentration increments (10^{-9} – 10^{-4} M). The arterioles were allowed to stabilize for 4 min at each drug concentration. In the second series, responses of the arterioles to cilostazol were examined during extraluminal administration of cyclo-oxygenase inhibitor, indomethacin (10 μ M) or NO synthase inhibitor, N^{ω} -nitro-L-arginine methyl ester (L-NAME, 30 μ M) or after chemical denudation of endothelium with Triton X-100 (0.01%) [22]. The concentration of indomethacin and L-NAME was high enough to eliminate the arachidonic acid-induced vasodilation or ACh-induced NO-dependent vasodilation, respectively [22]. Successful denudation of the endothelium was confirmed by loss of vasodilation in response to 10 μ M ACh. Indomethacin and L-NAME were suffused for at least 30 min before the cumulative administration of cilostazol commenced. In the third series, dose-response curves for cilostazol were obtained in the presence of ACh, isocarbacyclin, or SMP at a concentration that induces threshold-level vasodilation (100 nM, 1 nM, and 3 nM, respectively). The experimental procedure was the same as described in the first series of experiments. At the end of each experiment, all arterioles were relaxed completely with 10 μ M nifedipine to obtain maximum diameter at 60 mmHg intraluminal pressure.

Drugs and solutions. The composition of the MOPS solution (in mM) was as follows: 145 NaCl, 4.7 KCl, 2.0 CaCl_2 , 1.17 MgSO_4 , 2.0 pyruvate, 5.0 glucose, 0.02 EDTA, and 2.0 MOPS. The pH was adjusted to 7.4 ± 0.02 at 37°C . The solution used for dissection, cannulation, and perfusion was MOPS containing 1% BSA. Cilostazol was donated by Otsuka Pharmaceutical Co. (Tokyo, Japan). ACh chloride and isocarbacyclin were obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan) and Teijin (Tokyo, Japan), respectively. Indomethacin, L-NAME hydrochloride, and Triton X-100 were obtained from Sigma Chemi-

cal (St. Louis, MO, USA). Cilostazol was dissolved in N,N-dimethylformamide, and indomethacin and isocarbacyclin were dissolved in ethanol; these drugs were diluted with MOPS solution just before use. Each solvent at the final experimental concentration was confirmed to produce no significant effect on the diameter of pressurized arterioles. The other drugs were directly dissolved in the MOPS solution.

Statistics. Experimental data in the text, figures, and table are expressed as means $\pm$ standard error of mean (SEM). The diameter changes induced by agonists were normalized to the maximum dilation induced by nifedipine (10 μ M) from the control diameter and are expressed as a percentage of the nifedipine-induced maximum dilation. Control diameter was defined as the diameter immediately before determination of arteriolar response to vasoactive agents. From one animal, one to three vessel segments were dissected for study. The n value represents the number of vessel preparations used. The pD_2 value means the negative logarithm of ED_{50} , the concentration of agonists causing one-half maximum dilation. The vasodilation to various agonists and vasodilation before and after pharmacological interventions were analyzed with repeated measures analysis of variance (ANOVA) followed by Scheffe's test. Comparison of basal diameter before and after pharmacological treatments was performed with paired Student's t -test. A value of $p < 0.05$ was considered significant.

RESULTS

The passive diameters of spinal arterioles at 60 mmHg internal pressure were 110.8 ± 2.2 μm ($n=31$). After equilibration, arterioles developed myogenic tone and were constricted to $62.3 \pm 1.2\%$ of their maximum (passive) diameter.

Relaxant properties of spinal arterioles to vasoactive agents

Figure 2 represents typical spinal arteriolar dilation in response to cilostazol, and Fig. 3 demonstrates dose-response curves for the four vasodilator substances. Administration of cilostazol, ACh, isocarbacyclin, and SNP all produced dose-dependent vasodilations. The difference between the two concentration-response curves was statistically significant in all of the pairs among the four agonists ($p < 0.01$). The order of potency was found to be isocarbacyclin > ACh > SNP > cilostazol. The E_{max} and pD_2 values for these agents are summarized in Table 1.

Effects of indomethacin, L-NAME, or removal of the endothelium on cilostazol-induced arteriolar dilation

The effects of 10 μ M indomethacin or 30 μ M L-

Control

10 μ M Cilostazol

100 μ M Cilostazol

Fig. 2. Typical responses of a pressurized rabbit spinal arteriole to cumulative administrations of cilostazol. In this preparation, initial internal diameter before the pharmacological intervention was 72 μ m (control). During administration of 10 and 100 μ M cilostazol, vessel diameter increased to 90 and 96 μ m, respectively. Bar: 50 μ m.

NAME on cilostazol-induced vasodilation in the pressurized spinal arterioles are summarized in Fig. 4A, B. Incubation with indomethacin or L-NAME did not significantly alter the baseline diameter (65.6 ± 1.7 μ m before vs. 61.8 ± 3.2 μ m after indomethacin, and 66.8 ± 2.1 μ m before vs. 65.4 ± 2.8 μ m after L-NAME; $n=5$, respectively), and neither treatment significantly influenced the dose-response curve for cilostazol. Chemical denudation of the endothelial layers with 0.01% Triton X-100 caused a significant decrease in the baseline diameter, 13% (67.0 ± 3.1 μ m before vs. 58.3 ± 3.5 μ m after denudation, $p<0.01$; $n=4$). However, the removal of endothelium had a negligible effect on the cilostazol-induced arteriolar vasodilation as shown in Fig. 4C.

Effects of ACh, isocarbacyclin, or SNP

The effects of threshold levels of ACh, isocarbacyclin or SNP are summarized in Fig. 5A–C. Treatment with 100 nM ACh slightly, but significantly, increased the baseline diameter (66.6 ± 5.2 μ m vs. 69.2 ± 5.7 μ m, $p<0.05$; $n=5$). The presence of ACh, however, did not affect the concentration-response curves produced by cilostazol (Fig. 5A). Isocarbacyclin (1 nM) also increased the baseline diameter (63.1 ± 3.4 μ m vs.

Fig. 3. The dilator responses to cilostazol ($n=16$), acetylcholine (ACh, $n=5$), isocarbacyclin ($n=5$), and sodium nitroprusside (SNP, $n=5$) in pressurized spinal arterioles. Values are means $\pm$ SE.

Table 1. The $E_{\max}$ and pD_2 values for cilostazol, acetylcholine (ACh), isocarbacyclin, and sodium nitroprusside (SNP) in pressurized rabbit spinal arterioles.

Agonist (<i>n</i>)	Resting diameter (μ m)	Passive diameter (μ m)	$E_{\max}$ (%)	pD_2
Cilostazol (16)	65.9 ± 2.7	107.2 ± 2.8	74.3 ± 2.5	4.7 ± 0.1
ACh (5)	66.8 ± 2.9	113.2 ± 6.1	$82.5 \pm 3.4^{**}$	$5.6 \pm 0.0^{**}$
Isocarbacyclin (5)	72.4 ± 4.0	110.2 ± 5.3	$96.9 \pm 3.1^{**}$	$7.5 \pm 0.1^{**}$
SNP (5)	79.8 ± 10.4	120.6 ± 6.5	$95.3 \pm 2.0^{**}$	$7.0 \pm 0.1^{**}$

Values are mean $\pm$ SE. *n*, number of isolated arterioles. $E_{\max}$ is expressed as % of nifedipine-induced maximum dilation. $^{**}p<0.01$ vs. the value for cilostazol.

Fig. 4. Concentration–response curves for cilostazol in pressurized spinal arterioles before (○) and during (●) pharmacological blockade of cyclooxygenase or nitric oxide synthase, or before (○) and after (●) chemical denudation of endothelium. **A:** Before and during treatment with indomethacin (10 μ M, $n=5$). **B:** Before and during incubation of N^G -nitro-L-arginine methyl ester (L-NAME; 30 μ M, $n=5$). **C:** Before and after chemical denudation of endothelium with Triton X-100 (0.01%, $n=4$). Values are means $\pm$ SE.

Fig. 5. Concentration–response curves for cilostazol in pressurized spinal arterioles in the absence (○) or presence (●) of ACh (100 nM, $n=5$), isocarbacyclin (1 nM, $n=7$), or SNP (3 nM, $n=4$); each concentration of which produces a threshold level of vasodilation. **A:** Before and during presence of ACh. **B:** In the absence and presence of isocarbacyclin. **C:** Before and during administration of SNP. Values are means $\pm$ SE.

$65.3 \pm 3.6 \mu\text{m}$, $p < 0.01$; $n=7$). Isocarbacyclin slightly shifted the concentration–response curve for cilostazol to the left as shown in Fig. 5B, but statistical analysis revealed no significant difference between the two curves. SNP also produced a slight increase in baseline diameter, though it was not statistically significant ($66.6 \pm 5.2 \mu\text{m}$ vs. $69.2 \pm 5.7 \mu\text{m}$; $n=4$). SNP had no discernible influence on cilostazol-induced vasodilation in the spinal arterioles (Fig. 5C).

DISCUSSION

Our major findings in the present study are summarized as follows: (1) Cilostazol exerts mild vasodilation of pressurized arterioles isolated from rabbit spine in a concentration-dependent manner; (2) the drug produces vasodilation even during inhibition of cyclooxygenase or NO synthase, or after the chemical denudation of endothelial cells; and (3) simulating the release of endogenous vasodilators with ACh, or the administration of a prostacyclin analogue or a NO donor does not modify the efficacy of cilostazol. These results suggest that cilostazol produces vasodilation independent of the presence or activation of the endothelium in rabbit spinal arterioles. To the best of

our knowledge, this is the first demonstration of the effect of cilostazol in pressurized arterioles *in vitro*.

Vasodilator action of cilostazol on resistance vessels.

Cilostazol produced a dose-dependent dilation of pressurized spinal arterioles, although the dilator effect was mildest among the four vasodilators studied. Previously, cilostazol was shown to increase blood flow in the finger of diabetic patients with peripheral vascular disease, as evidenced by an increase in digital skin temperature [23]. In anesthetized dogs, cilostazol decreases blood pressure by reducing resistance in the peripheral blood vessels [24]. These findings support our conclusion that cilostazol can act directly on the resistance-sized vessels to alter peripheral blood flow. A recent clinical trial showed that cilostazol significantly reduces the recurrence of lacunar infarction, which involves occlusion of resistance-sized vessels in the brain [9]. Another study also indicated that long-term oral administration of cilostazol improved cerebral blood flow in patients with cerebral infarction [11]. Our findings in the present study support the idea that the vasodilator action of cilostazol at the arteriolar level may, at least in part, contribute to the beneficial effect of cilostazol preventing secondary cerebral infarction.

Mechanisms of arteriolar dilation with cilostazol: negligible modulation with endothelium, endogenous nitric oxide, and prostaglandins. The present study indicates that cilostazol exerts the vasodilation of pressurized spinal arterioles independent of endogenous nitric oxide and vasodilator prostaglandins since indomethacin, L-NAME, and even chemical denudation of endothelial cells did not significantly modify the response. The concentration of inhibitors used in the present study is high enough to block cyclo-oxygenase and nitric oxide synthase activity since the co-administration of 10 μ M indomethacin and 30 μ M L-NAME completely eliminated ACh-induced dilation of the spinal arterioles [22]. Nitric oxide activates soluble guanylate cyclase in vascular smooth muscles and increases cGMP in the cells. The increased level of cGMP could inactivate PDE3, thereby increasing the cAMP concentration in the cells and causing further vasodilation. Endogenous nitric oxide may thus compete with cilostazol against the PDE3 inhibition and reduce the efficacy of cilostazol. Vasodilator prostaglandins, on the other hand, increase the cAMP in smooth muscles via activating of adenylate cyclase [15–17]. Thus, endogenous prostanoids may potentially increase the dilator effect of cilostazol. Igawa *et al.* [25] demonstrated a synergistic action of cilostazol with endothelium on anti-platelet aggregating activity, and aspirin reversed the potentiated inhibitory effect of cilostazol. Such potentiation between cilostazol and endothelium on arteriolar dilation, however, was not apparent in the present study. Our results are in agreement with the report by Shigemori *et al.* [18] that cilostazol exerts vasodilation in the rat pulmonary arterial ring precontracted with phenylephrine regardless of the endothelium function. On the other hand, Nakamura *et al.* [26] recently suggested that the vasodilator action of cilostazol is partially endothelium dependent, and that NO production is increased with cilostazol in the rat thoracic aorta.

Our results may suggest that endogenous vasodilators from intact endothelium released at rest are not high enough to modulate the action of cilostazol. To address this issue, we attempted to stimulate endothelium with a low dose of ACh to increase the release of endogenous endothelium-derived relaxing factors. We also tried to increase the basal level of cAMP or cGMP in the smooth muscles via the administration of a prostacyclin analogue or a NO donor. None of these treatments, however, was effective in modulating cilostazol-induced arteriolar dilation. These results may suggest that the vasodilator effect of cilostazol cannot be solely explained by PDE3 inhibition. The

idea is, in part, supported by the report of Cone *et al.* [27], in which they compared the effects of cilostazol and milrinone, a conventional PDE3 inhibitor, on changes in cAMP levels and functions in platelets, ventricular myocytes, and coronary smooth muscle cells of rabbits. They showed that, in coronary smooth muscle cells, milrinone elevates cAMP and increases coronary blood flow; however, cilostazol did not increase intracellular cAMP while producing a comparable increase in the coronary flow. They also showed that, in rabbit ventricular myocytes, cilostazol does not increase cAMP as much as milrinone does, and that the cardiotropic effect of these agents is significantly less with cilostazol. Similarly, Tanaka *et al.* [28] did not see an increase in intracellular cAMP after a 12-min exposure of rabbit aorta smooth muscle cells to 10 μ M cilostazol. Lindgren *et al.* [29] compared OPC-3911 (a PDE3 inhibitor structurally similar to cilostazol) with milrinone in rat aorta, and found the increase was significant only for milrinone. Nevertheless, OPC-3911 and milrinone had similar relaxant properties in human and rat coronary and renal arteries [30]. The data may not mean less potency of cilostazol on PDE3 inhibition, because cilostazol and milrinone concentration-dependently increased intracellular cAMP in platelets and inhibited human platelet aggregation with similar potency [27]. These findings, including ours, may support the conclusion that the anti-PDE3 effect of cilostazol is, to some extent, cell specific and that the detailed mechanisms of cilostazol action on the vascular smooth muscle remain to be elucidated.

The authors thank Mr. Nobuyuki Ono, Nagano Technical College, for his technical support. This work was supported in part by the Research Grant for Cardiovascular Diseases (9 koubo 3) from the Ministry of Health and Welfare of Japan and by a Grant-in-Aid for Scientific Research (No. 13770023) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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