Cilostazol Increases Tissue Blood Flow in Contracting Rabbit Gastrocnemius Muscle

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Background: The mechanisms underlying the ability of cilostazol to improve walking distance in patients with intermittent claudication (IC) are not fully understood, but may be related to its phosphodiesterase type 3 (PDE3) and adenosine uptake inhibition. In the present study the effect of cilostazol on blood flow and interstitial adenosine concentration was compared with that of the PDE3 inhibitor, milrinone, and the adenosine uptake inhibitor, drafflazine.

Methods and Results: Rabbit gastrocnemius muscle blood flow was measured under resting, contracting and ischemic conditions. Interstitial adenosine was sampled by microdialysis. None of the drugs affected tissue blood flow at rest. Blood flow in electrically stimulated muscle was 2- to 3-fold higher in vehicle-, milrinone- and drafflazine-treated animals. However, cilostazol caused an 8-fold increase. Ligation of the femoral artery decreased blood flow in the stimulated muscle in all groups to a similar degree. Cilostazol and drafflazine increased the dialysate adenosine concentration during the first 10 min of muscle contraction, but had no effect during ischemia, most likely because of the high AMP deaminase activity in skeletal muscle.

Conclusions: Cilostazol increases blood flow in the gastrocnemius muscle during contraction and it is this effect that may be partially responsible for the improved walking distance in IC patients. (Circ J 2010; 74: 181–187)

Key Words: Adenosine; Blood flow; Cilostazol; Intermittent claudication; Phosphodiesterase type 3

Cilostazol (Pletal®) has been used in Japan and other Asian countries (as Pletaal®) to treat the symptoms of lower extremity peripheral arterial disease (PAD). In the US and several European countries, cilostazol is approved for treatment of the symptoms of intermittent claudication (IC), the most common debilitating symptom of PAD. IC causes muscle pain and a reduced capacity for exercise, resulting in a marked impairment in quality of life and daily activities. The mechanisms by which cilostazol exerts its beneficial effects are not fully understood, but most likely are the consequences of antiplatelet and vasodilatory effects. Inhibition of vascular smooth muscle cell proliferation and improvement of the lipid profile may also play beneficial roles. Pharmacologically, cilostazol is a dual inhibitor, inhibiting both cyclic nucleotide phosphodiesterase type 3 (PDE3) and adenosine uptake. PDE3 inhibition elevates intracellular cAMP and the inhibition of adenosine uptake elevates interstitial and circulatory adenosine concentration. In platelets and vascular smooth muscle cells, adenosine increases intracellular cAMP by activating adenosine A2 receptors. Simultaneous inhibition of PDE3 and elevation of extracellular adenosine causes a synergistic increase in the concentration of intraplatelet cAMP, resulting in a potent antiplatelet effect. Although cilostazol’s well-documented antiplatelet effect is known to benefit IC patients, peripheral vasodilation may also contribute to the improvement in walking distance, but there are few studies of cilostazol’s effect on skeletal muscle blood flow. Moreover, in that limited number of studies, blood flow was determined in resting muscle, whereas the symptoms of IC occur only during exercise, so effects observed at rest may not necessarily be duplicated during exercise.

In this study, we used a fluorescent microsphere method to investigate cilostazol’s effect on blood flow in resting rabbit gastrocnemius muscle, during electrically stimulated contraction (to simulate walking), and under ischemic conditions, induced by ligation of the femoral artery. We also measured interstitial adenosine levels using microdialysis and the levels of AMP deaminase (AMPD), a rate-limiting enzyme of purine catabolism, in cardiac and skeletal muscle.

Methods

The present study was conducted in accordance with the...

Chemicals
Cilostazol and draflazine were provided by the Otsuka Pharmaceutical Co Ltd, Japan. All other chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA).

Surgical Preparations
Male rabbits (New Zealand White), weighing 2.5–3.5 kg, were anaesthetized with intravenous pentobarbital (30 mg/kg), administered via the marginal ear vein. A tracheotomy was performed and the animals were intubated. Ventilation was with room air supplemented with 100% O2 via a Harvard small animal ventilator (Harvard Apparatus, Holliston, MA, USA). The respiratory rate was adjusted to keep arterial blood PO2, PCO2 and pH in the physiological range. Body temperature was maintained at 38±1°C with a heating blanket. The jugular vein was cannulated for additional anesthetic and drug administration. A Millar pressure transducer (4F; Miller Instruments, Houston, TX, USA) with lumen was inserted into the left carotid artery and advanced to the left ventricle for infusion of fluorescent microspheres. The right carotid artery was cannulated for arterial blood pressure measurement. The femoral artery of each hindlimb was exposed through a longitudinal skin incision in the medial thigh, extending from the inguinal ligament to the stifle. Arterial occlusion and reperfusion were achieved by the placement and removal, respectively, of an artery clamp. After the gastrocnemius muscles of both hindlimbs were surgically exposed, a 10-mm linear microdialysis probe (Bioanalytical Systems, West Lafayette, IN, USA) was implanted in the mid-gastrocnemius muscle, perpendicular to the direction of the muscle. To stimulate muscle contraction, a pair of electrodes was placed on the sciatic nerve of the left hindlimb and connected to a Grass SD9 stimulator. The stimulation was produced with an 8-ms square supra-maximal pulse of 10 V at 1 Hz. The feet were positioned perpendicularly to the leg. The contralateral hindlimb served as a control and was not stimulated.

Determination of Plasma Concentrations of Cilostazol and Milrinone
The plasma concentrations of cilostazol and milrinone were determined by high-performance liquid chromatography using methods described by Fu et al15 and Verrijk et al,16 respectively. The blood samples were drawn from separate groups of 4 animals for each treatment to determine the plasma concentration of the infused drug. Infusion protocols, as described next, were then established to achieve clinically relevant plasma concentrations.

Experimental Protocol
The time course of the experiment is shown in Figure 1. At 60 min after the surgical preparation, animals were divided into 4 groups and received either dimethyl sulfoxide (DMSO as vehicle) or a test compound. The first group (n=6) was the control group and received a bolus of 0.3 ml/kg DMSO followed by 1 μl·kg⁻¹·min⁻¹ for 150 min. The 2nd group of rabbits (n=6) received cilostazol as a bolus of 1 mg/kg followed by 60 μg·kg⁻¹·min⁻¹ for 150 min. The 3rd group of rabbits (n=6) was treated with milrinone, a PDE3 inhibitor, administered as a bolus of 0.2 mg/kg followed by a continuous infusion of 10 μg·kg⁻¹·min⁻¹ for 60 min, followed by 5 μg·kg⁻¹·min⁻¹ for 90 min. Finally, draflazine, an adenosine uptake inhibitor, was given to the 4th group of rabbits (n=6) as 2 bolus doses of 0.1 mg/kg given at 0 min and 60 min (Figure 1). We selected the intravenous infusion protocol for cilostazol in order to produce a sustained blood plasma level of 3–5 μmol/L, which is similar to the therapeutic concentration in humans, with a Cmax of ≈3 μmol/L.17 The infusion protocol used for milrinone produced a sustained plasma concentration of 3–6 μmol/L. The dose of draflazine was based on previous studies in which it was shown that a 1-mg/kg intravenous bolus inhibits more than 70% of adenosine uptake for up to 60 min.18,19

Stimulation of the left gastrocnemius was initiated 30 min after the start of DMSO or drug infusion and continued throughout the remainder of the experiment. At 30 min after the onset of stimulation (60 min time point in Figure 1), the left femoral artery was clamped for 60 min and released to allow reperfusion. Regional blood flow was determined by the injection of blue-green, yellow-green, orange, red and crimson fluorescent microspheres at 0, 30, 60, 120, and 150 min, respectively (red circles in Figure 1). Regional Blood Flow Determination
Blood flow was measured using fluorescent microspheres according to the “Manual for using fluorescent microspheres to measure organ perfusion” (Fluorescent Microsphere Resource Center, University of Washington, Seattle, WA, 1999). Blue-green, yellow-green, orange, red and crimson

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**Figure 1.** Experimental protocols. Fluorescent microspheres were injected at 0, 30, 60, 120, and 150 min to determine regional blood flow.
fluorescent-labeled polystyrene microspheres (15-μm diameter) were purchased from Molecular Probes (Eugene, OR, USA) and 5×10^6/kg of body weight of each type of fluorescent microsphere was injected into the left ventricle through a catheter in 20 s. Simultaneously, a blood sample was withdrawn from the right carotid artery at 2.5 ml/min for 2 min, starting 30 s before the injection of the microspheres. At the end of the experiment, the rabbit was killed humanely with a lethal dose of sodium pentobarbital (100 mg/kg). Tissue samples (~1 g each) were taken from the left ventricular free wall, the left kidney, and the gastrocnemius muscle of both hindlimbs. The samples were weighed, placed in tubes and processed for digestion and fluorimetry. Fluorescence was measured with a spectrofluorometer (Fluomax-2, Instruments S.A., Edison, NJ, USA). Regional blood flow was calculated by the standard reference flow technique,20 and expressed as ml·min⁻¹·100 g⁻¹.

**Statistical Analysis**
Data are presented as mean±SEM. The data were analyzed by 2-way (group and time as variances) ANOVA (analysis of variance) with repeated measurements followed by a post hoc Student-Newman-Keuls test (SigmaStat 3.10.0, Jandel Corporation, San Rafael, CA, USA). P<0.05 was taken as
the level of statistical significance.

Results

The basal hemodynamic parameters of the 4 groups were similar (Table 1). After 30 min of drug treatment, milrinone significantly increased heart rate (HR), and cilostazol and milrinone decreased mean arterial blood pressure (MAP). Although draflazine did not alter HR, it decreased MAP. Muscle stimulation did not have additional effects on HR or blood pressure. Cilostazol, milrinone and draflazine all increased blood flow in the left ventricle, although the changes were not significant. Cilostazol and draflazine slightly increased, whereas milrinone slightly decreased, renal blood flow. During muscle stimulation, blood flow in the left ventricle and skeletal muscle tended to decrease in all treatment groups, although none of the changes reached statistical significance.

Drug treatment alone did not significantly alter blood flow in the resting gastrocnemius muscle of either leg (Figure 2), whereas stimulation significantly increased flow in the affected muscle. Cilostazol alone further increased muscle blood flow during stimulation (from 4.3±1.1 to 38.5±6.2 ml·min⁻¹·100 g⁻¹, P<0.03 vs DMSO-, milrinone-, or draflazine-treated). Ligation of the left femoral artery greatly reduced blood flow in the gastrocnemius muscle to a similar extent in all groups, and flow was partially recovered during reperfusion, irrespective of the treatment. Gastrocnemius muscle completely ceased contraction in all the animals 10 min after the start of ischaemia and did not recover during reperfusion, with the exception of those receiving cilostazol in which some return of contraction was observed in 3 of 6 animals.

In the control muscle, interstitial adenosine levels did not change throughout the experiment (Figure 3). Stimulation slightly (although not significantly) increased adenosine levels in DMSO- and milrinone-treated muscles (Figure 3). Both draflazine and cilostazol significantly elevated the adenosine concentration during the onset of muscle stimulation (from 0.07±0.01 μmol/L at baseline to 0.22±0.05 μmol/L for draflazine and from 0.06±0.01 μmol/L at baseline to
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0.21±0.06μmol/L for cilostazol, 10 min after the start of the stimulation), but the increase was not sustained and after 30 min of stimulation adenosine levels were similar among all groups. Femoral artery occlusion did not elevate the interstitial adenosine concentration. AMPD and adenosine deaminase (AD) activities were measured in samples taken from the gastrocnemius and left ventricular muscles. As shown in Table 2, in DMSO-treated animals AMPD activity was approximately 85-fold higher in the soluble fraction and 239-fold higher in the membrane-bound fraction in gastrocnemius muscle. AD activity, on the other hand, was approximately 4-fold higher in the soluble fraction and 3-fold higher in the membrane-bound fraction of the cardiac tissue than in the skeletal muscle. Ischemia did not affect AMPD and AD activities, except in cilostazol-treated animals, in which AMPD activity decreased in the soluble fraction and increased in the membrane fraction. AD activity was not significantly affected.

Discussion

Clinical studies conducted in the US show that cilostazol is effective in improving walking distance in IC patients (see reviews1.21), but the underlying mechanisms remain to be determined. Blood flow is sufficient to meet the resting requirements of IC sufferers, but when they exercise, skeletal muscle blood flow fails to increase adequately and there is a rapid onset of muscular pain and fatigue.22 Most often this is caused by atherosclerotic occlusive or stenotic lesions in the peripheral circulation. Cilostazol potently inhibits PDE3 (IC50: 0.2μmol/L) and modestly inhibits adenosine uptake (IC50: 5μmol/L). The inhibition of cellular adenosine uptake increases extracellular adenosine concentration. Through a synergistic interaction between PDE3 inhibition and activation of adenosine A2 receptors, cilostazol inhibits platelet aggregation and thrombosis formation.17-9 The majority of the pharmacological investigations of cilostazol has studied the inhibition of platelet activation and aggregation. Platelet activation plays an important role in initiating atherosclerosis, and thrombotic narrowing/occlusion of peripheral blood vessels may contribute to the symptoms of IC.21 Although improving blood circulation in IC patients during walking has been a target for pharmacotherapy, very few studies have considered the peripheral vasodilatory effect of cilostazol. In an original paper describing the discovery of cilostazol, Kawamura et al found that it caused a dose-dependent, broad vasodilation of the common and internal carotids, as well as the vertebral, superior mesenteric and femoral arteries, in an artificial constant perfusion system.12 Plasma concentration was not measured in that study, so it is unknown whether their observed effect is clinically significant. Birk et al found that cilostazol dilated the large cerebral arteries without affecting regional cerebral blood flow in human subjects.23 In patients with arteriosclerosis obliterans of the lower extremities, 2 weeks of treatment with cilostazol (100 mg bid) significantly increased tissue blood flow during reactive hyperemia measured by the venous occlusion method using a strain-gauge plethysmograph.26 We show here that cilostazol does not affect blood flow in resting gastrocnemius muscle; however, an augmented elevation of blood flow was observed in electrically stimulated muscle when the animal was treated with cilostazol, but not with milrinone or drafazine. These findings suggest that cilostazol’s effect on gastrocnemius muscle blood flow is unlikely to be related to either PDE3 inhibition or adenosine uptake inhibition alone. Logically, it would be interesting to determine whether a combination of milrinone and drafazine can mimic the effect of cilostazol. However, a profound hypotensive effect was observed in our preliminary testing when milrinone and drafazine were co-administered. Future pharmacokinetic studies are needed to find a combination that will inhibit PDE3 and adenosine uptake to a similar degree as cilostazol, and then examine the effect of that combination.

IC patients often have underlying atherosclerotic disease and narrowing of arteries, and may have diabetes mellitus; these conditions were not reproduced in our model. In future, it may be worthwhile studying gastrocnemius blood
flow and adenosine level in atherosclerotic animals with partial stenosis, as it more closely mimics the conditions in the legs of ambulatory IC patients.

Costa et al reported that intermittent dynamic exercise increased the interstitial adenosine concentration in human flexor digitorum superficialis muscle in an exercise intensity-dependent fashion. In the present study, we observed a small but statistically insignificant increase in interstitial adenosine in the electrically stimulated gastrocnemius muscle. Cilostazol and drafazine both elevated adenosine during the initial muscle contraction, but mhirnane had no effect, suggesting the elevation of adenosine was caused by inhibition of adenosine uptake. The increase in adenosine was not sustained, however. Surprisingly, adenosine concentration was not elevated during ischemia, even with adenosine uptake inhibitors; this finding is in contrast with the well-documented elevation of adenosine in the ischemic heart. We believe that variations in the purine metabolism of cardiac and skeletal muscle may contribute to this difference. When energy demand is increased, or supply is reduced, adenosine is produced from the catabolism of ATP to AMP. AMP is then either de-phosphorylated by 5'-nucleotidase to form adenosine or deaminated by AMPD to give IMP (Figure 4). AMPD is the rate-limiting enzyme for entry into the purine nucleotide cycle and catalyzes the conversion of AMP to IMP. Alternatively, AMP is converted to adenosine by 5'-nucleotidase. AMPD activity is known to be significantly higher in skeletal muscle compared with cardiac muscle, an observation confirmed in our current study. In skeletal muscle, because the majority of AMP is degraded via AMPD, thus bypassing the adenosine-producing step, the amount of adenosine produced is small. However, even with high AMPD activity, ischemia should lead to an increase in adenosine production. Thus it is puzzling that in our study the interstitial adenosine level was not elevated during ischemia. One possible explanation is that the ATP store may have been depleted soon after the onset of ischemia; this is suggested by the cessation of visible muscle contraction during the first 10 min of ischemia and minimal or no recovery of contraction during reperfusion.

Cilostazol slightly increased AMPD activity in our study (Table 2), most likely because of PDE3 inhibition and elevation of cAMP, as protein kinase A has been reported to increase AMPD activity. In the cilostazol-treated ischemic muscle, AMPD activity in the soluble fraction was reduced, but was higher in the membrane. Ischemia is known to activate protein kinase C, which can also increase AMPD activity. Thus the interaction between protein kinases A and C likely contributes to the changes in AMPD. Because cilostazol did not change blood flow during ischemia, any significant AMPD modification requires further investigation in different models.

It is unclear which mechanisms may be responsible for the observed increase in blood flow in contracting gastrocnemius muscle. The role of adenosine is not certain, because the increase in adenosine during stimulation was relatively small. One potential contributor may be nitric oxide (NO). In a study by Nakamura et al, cilostazol induced relaxation of thoracic aorta, pre-contracted by phenylephrine, in a concentration-dependent manner. The concentration-dependent relaxation was shifted to the right in denuded aorta, compared with intact endothelium, suggesting that this relaxation was partly dependent on endothelium. Cilostazol also significantly increased the NO level in porcine thoracic aorta, and relaxation was reversed by treatment with a competitive inhibitor of NO synthase. Similar findings have been reported for human aortic endothelial cells. Future studies are needed to explore the potential contribution of NO to the observed increase in blood flow by cilostazol.

In summary, we have shown that cilostazol increases tissue blood flow in exercising gastrocnemius muscle and this effect may be partially responsible for the improvement in the walking distance of IC patients treated with cilostazol. However, the mechanisms for this increase remain to be fully elucidated.

Acknowledgments

We thank Maurice Guertin, Marina Samuel and Kevin Lee for their support with adenosine measurement, and determination of plasma concentrations of cilostazol and mhirnane. We also thank Simon Lockyer for assistance with this manuscript.

Disclosure

All the authors are employees of the Otsuka Maryland Medicinal Laboratories Inc, a company wholly owned by the Otsuka Pharmaceutical Group, which is currently marketing or has marketed cilostazol as Pletal™ in the EU and USA, and as Pletaal™ in Japan and Asian countries.

References

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