Effect of cilostazol on experimental diabetic neuropathy in the rat

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Summary Two proposed mechanisms of diabetic neuropathy are microvascular ischaemia and a reduction in Na,K-ATPase activity. We evaluated the effect of cilostazol, a drug that is both a potent phosphodiesterase inhibitor that normalizes nerve Na,K-ATPase and a vasodilator, on nerve blood flow (NBF) to determine whether it would improve experimental diabetic neuropathy. We examined whether epineurally applied cilostazol acted as a vasodilator on the peripheral nerve of normal and diabetic rats, and whether feeding the rats a cilostazol-supplemented diet could improve diabetic neuropathy. Cilostazol increased nerve blood flow (NBF) in a dose-dependent fashion with an EC_{50} of $10^{-5.74}$ mol/l.

Cilostazol also normalized NBF in experimental diabetic neuropathy with a 10^{-4} mol/l local application on the sciatic nerve. In diabetic neuropathy, a cilostazol-supplemented diet improved both NBF and nerve conduction in a dose- and time-dependent fashion. Potential mechanisms of action of cilostazol on the nerve include its effect on NBF, Na, K-ATPase, and restoration of the thromboxane:prostacyclin ratio. Cilostazol may have potential in the treatment of diabetic neuropathy. [Diabetologia (1995) 38: 914–918]

Key words Diabetic neuropathy, nerve blood flow, vasodilator, sciatic nerve, cilostazol.

In experimental diabetic neuropathy (EDN), nerve blood flow (NBF) is reduced and the oxygen tension histogram is shifted into the hypoxic range [1]. Moreover, hypoxia per se, in otherwise normal nerves, will reproduce much of the lipid and electrophysiologic abnormalities of EDN [2]. Therapeutically, increasing the oxygen supply by supplementation or hyperbaric oxygenation has been shown to prevent and reverse nerve electrophysiology [3, 4]. An alternative approach is to increase nerve blood flow by chemical

sympathectomy [5], vasodilators, such as α -antagonists [5], or eicosanoids [6]. Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone) is a potent phosphodiesterase inhibitor [6, 7]. It blocks platelet aggregation and is a vasodilator of cerebral and femoral arteries [8, 9]. Its effects on peripheral nerves are unknown. We tested the hypothesis that cilostazol may be a peripheral nerve vasodilator, and that it might be effective in improving reduced NBF in EDN. We evaluated both the topical application (to elucidate its mode of action, and establish a dose-response relationship), and oral feeding in the prevention of NBF and conduction abnormalities in EDN.

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Abbreviations: EDN, Experimental diabetic neuropathy; NBF, nerve blood flow; STZ, streptozotocin; NRC, control rats receiving normal diet; NRH, control rats receiving a high (0.1 %) cilostazol diet; CSH, STZ rats receiving high (0.1 %) cilostazol diet; CSL, STZ rats receiving low (0.03 %) cilostazol diet; CV, conduction velocity.

Materials and methods

Animals. We used male Sprague Dawley rats with initial weights of about 250 g. These animals were housed in cages with plastic floors covered with wood shavings and were fed

Purina rat chow (no. 5001; Purina, St. Louis, MO, USA) with an unrestricted supply of water.

Experimental diabetes was induced with streptozotocin (STZ), administered by intraperitoneal injection. STZ was dissolved in 0.05 mol/l sodium citrate at pH 4.5 (65 mg/ml; dose = 1.32 ml/kg). Rats were presumed diabetic if their fasting blood glucose level exceeded 16.7 mmol/l 7 days after injection of STZ and remained greater than 16.7 mmol/l throughout the study.

We evaluated the effects of the epineural application of cilostazol on sciatic NBF in normal rats in order to determine whether it had a vasodilator action, and if it could normalize the reduced NBF of EDN and to obtain a dose-effect relationship. We also evaluated the effect of oral cilostazol supplementation in reversing STZ-induced EDN. Cilostazol was provided as a gift by Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan.

Local application. We used a previously described nerve preparation [10]. In brief, rats were anaesthetized with an i.p. injection of barbiturate (pentobarbital; 100 mg/kg), paralysed with curare (2 U i.v. and 6 U i.p.), and ventilated using a rodent respirator supplying a mixture of nitrogen and oxygen to maintain arterial blood gases within the physiological range (pH 7.4 ± 0.5 ; pO_2 110 ± 20 ; pCO_2 40 ± 5 mmHg).

The nerve preparation has been especially modified for the study of vasoactive agents on peripheral nerve [10]. A length of left sciatic nerve not exceeding 2 cm was exposed and a pool was formed by the surrounding muscle and skin. The pool was filled with mammalian Ringer's solution and the temperature was maintained at $33.5 \pm 0.1^\circ\text{C}$ using a servo-controlled infrared heat lamp. Mammalian Ringer's solution was composed of: (in mmol/l) Na 145, K 3.5, Ca 2, glucose 6 and Hepes buffer 2 and adjusted to pH 7.4. Rectal temperature was maintained between 36.5 and 37.5°C by infrared lamp. The nerve was superfused with solutions of known concentrations of cilostazol. The effects of cilostazol at concentrations of 10^{-9} , 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} mol/l were examined in a normal nerve and at concentration of 10^{-4} mol/l in a diabetic nerve. The rat was killed with an overdose of pentobarbital at the end of the study.

Cilostazol supplementation study. We separated rats into five groups. Each group received their respective diets for a total of 4 weeks.

- (1) Controls receiving normal diet (NRC), $n = 6$
- (2) Controls receiving a high (0.1 %) cilostazol diet for 4 weeks (NRH), $n = 7$
- (3) STZ receiving normal diet (STZ), $n = 5$
- (4) STZ receiving high (0.1 %) cilostazol diet (CSH), $n = 6$
- (5) STZ receiving low (0.03 %) cilostazol diet (CSL), $n = 6$

To ensure that the cilostazol-supplemented rats were neuropathic, we performed nerve conduction studies in STZ rats before cilostazol feeding and only those rats with motor conduction velocities in sciatic-tibial nerve of 30 m/s or lower were used.

Measurement of NBF. NBF was measured by microelectrode hydrogen polarography (tip size 2–5 μm) [10]. The signal was input into a computer via an analogue-to-digital converter (Colbourn Instruments, Lehigh Valley, Pa., USA) for simultaneous display (Labtech Notebook Laboratory Technologies Corp., Cambridge, Mass., USA) and storage (Lotus 123, Lotus Development Corp., Cambridge, Mass., USA). A curve was fitted to the data using a non-linear regression program based on the Marquardt algorithm [11]. Records were taken from the centre of the fascicle. Blood pressure and heart rate were continuously monitored.

Electrophysiology. In the electrophysiological studies we used techniques that are standard for our laboratory. Motor conduction velocities and the amplitude of the compound muscle action potential were measured in the sciatic-tibial and caudal nerves [12]. Sensory conduction velocities and nerve action potential amplitude were measured in the caudal nerve [13]. Fine stainless steel near-nerve stimulating and recording electrodes were employed. Recordings were made at 35°C , amplified 1000 times, stored on computer discs, and analysed off-line using a Nicolet 4094 digital oscilloscope (Madison, WI, USA) with associated stimulators and stimulus isolation units.

Blood glucose level. Blood glucose level was determined at 4 weeks using glucostix reagent strips and a glucose meter (Ames Glucometer II, model 5550, Elkhart, IN, USA). Rats were presumed diabetic if their blood glucose level was greater than 19.6 mmol/l.

Statistical analysis

Statistical analysis was performed on a 486PC computer system with Systat for Windows (Systat Inc., Evanston, Ill., USA) statistical program. Curve fitting to derive EC₅₀ and asymptote were carried out using the Graphpad curve-fitting and plotting program (ISI Software, Philadelphia, Pa., USA). Group data were compared using two-tailed unpaired *t*-test. Values are expressed as mean $\pm$ SEM and significance was accepted when $p < 0.05$.

Results

Blood glucose level. The blood glucose values (mean $\pm$ SEM), at time of killing were as follows: NRC, 5.1 ± 0.2 ; NRH, 5.2 ± 0.3 ; STZ, greater than 22.5; CSH, greater than 22.5; CSL, greater than 22.5 mmol/l. The diabetic groups all had significantly higher blood glucose levels ($p < 0.001$) than controls and controls with high cilostazol diet.

Body weight. Body weight values at time of killing were as follows: NRC, 412.6 ± 11.3 ; NRH, 432.0 ± 23.5 ; STZ, 196.4 ± 20.2 ; CSH, 209.2 ± 26.3 ; CSL, 176.4 ± 22.1 g. The diabetic groups all had significantly lower weights ($p < 0.001$) than controls and controls with high cilostazol diet.

Dose-response study: epineurally applied cilostazol

(1) Normal nerve: A dose-response study was performed on the sciatic nerve of six control rats. Cilostazol was superfused at concentrations of 10^{-9} , 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} mol/l. Cilostazol increased NBF in a dose-dependent fashion (Fig. 1). Resting NBF was 14.3 ± 1.0 ml $\cdot$ 100 g $^{-1}$ $\cdot$ min $^{-1}$. A dose of 10^{-9} appeared to be the threshold dose and an increase of $52.7 \pm 16.9\%$ occurred with 10^{-4} mol/l. The EC₅₀ was $10^{-5.74}$ mol/l. Arterial blood pressure remained constant throughout the experiments. Corresponding

Fig. 1. Dose-response study relating nerve blood flow, expressed as percent change against log-molar concentration of epineurally applied cilostazol. Bars represent $\pm$ SEM

Fig. 2. Effect of epineurally applied cilostazol on sciatic nerve blood flow of experimental diabetic neuropathy

Fig. 3. The effect of oral supplementation of cilostazol for 4 weeks on sciatic nerve blood flow of control (NRC and NRH) and diabetic (STZ) rats in low (CSL) and high (CSH) concentration

values for nerve vascular resistance in $\text{mmHg} \cdot \text{ml}^{-1} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$ were 15.0 ± 0.6 , 9.6 ± 0.6 , 9.8 ± 0.8 , 7.5 ± 0.5 , and 6.9 ± 0.7 for 10^{-9} , 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} mol/l, respectively.

(2) *Diabetic neuropathy:* The diabetic state resulted in a significant reduction ($p < 0.05$) in NBF ($8.2 \pm 0.6 \text{ ml} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$) when compared with controls ($14.3 \pm 1.0 \text{ ml} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$). The reduction in flow

Fig. 4. The effect of oral supplementation of cilostazol for 4 weeks (4 W) on sciatic-tibial nerve conduction velocity (CV) on controls (NRC and NRH), diabetics (STZ) in low (CSL) and high (CSH) concentrations. B and 4 W refer to values before and 4 weeks after supplementation

was associated with a significant increase in microvascular nerve resistance. Both NBF and vascular resistance (not shown) were normalized with local application of 10^{-4} mol/l cilostazol (Fig. 2).

Oral supplementation of cilostazol on experimental diabetic neuropathy

Following 4 weeks of dietary manipulation, NBF was measured in the left sciatic nerve. This was followed by electrophysiological studies on the same animals. Blood was also drawn for determination of blood glucose level.

(1) *Nerve blood flow:* The STZ group had a significant reduction in NBF when compared with controls ($p < 0.05$). Cilostazol supplementation had no effect on control nerves, while its effect on diabetic nerves consisted of a modest non-significant improvement at low doses and improved NBF in the CSH group (STZ vs CSH, $p < 0.05$; NRC vs CSH, $p > 0.05$; Fig. 3).

(2) *Electrophysiology:* Sciatic-tibial nerve conduction velocity was significantly reduced in the diabetic animals (STZ vs NRC, $p < 0.05$). Cilostazol treatment improved nerve conduction velocity in both CSL ($p < 0.01$) and CSH ($p < 0.03$) (Fig. 4).

Caudal conduction velocity was reduced in the STZ group and became more abnormal with increasing duration of diabetes. Cilostazol treatment improved conduction velocity in a dose-dependent manner, reaching statistical significance in the CSH group ($p < 0.03$) (Fig. 5).

Fig. 5. The effect of oral supplementation of cilostazol for 4 weeks (4 W) on caudal nerve conduction velocity (CV) on controls (NRC and NRH), diabetics (STZ) in low (CSL) and high (CSH) concentrations. B and 4W refer to values before and 4 weeks after supplementation

Discussion

The main findings of this study are that cilostazol is a potent vasodilator of rat peripheral nerves, and that it will acutely normalize the reduction in NBF in EDN. Oral feeding will improve both the NBF and conduction slowing of EDN.

We used a combination of microelectrode polarography and topical superfusion to test this agent. This method has two major advantages. The first is the avoidance of a systemic effect, an important property, since the systemic effect of vasoactive agents tends to overwhelm the lesser effects on the nerve microvascular bed. The second advantage of our preparation is the ability of topically applied agents to rapidly bind to epineurial receptors, vascular smooth muscle and, because of a lack of a blood-nerve barrier, to endothelial receptors.

Cilostazol has several properties potentially beneficial to diabetic neuropathy. Vasodilator effects of cilostazol have been shown in the dog where the percent dilatation using cilostazol (100 ml/min) in the femoral artery, vertebral artery, internal carotid artery, and renal artery was 138.0 ± 19.5 , 74.4 ± 10.5 , 41.1 ± 6.2 and 7.1 ± 0.8 %, respectively [8]. Our results indicate that cilostazol has a direct vasodilator effect on epineurial arterioles of peripheral nerve producing similar effects to those seen in the vertebral artery. The magnitude of vasodilatation is similar to that achievable with phentolamine [14], guanethidine [5, 14], with the vasodilator peptides such as substance P and calcitonin gene related peptide [15], and vasodilator eicosanoids (Kihara and Low, unpublished data). The EC_{50} of $10^{-5.7}$ mol/l is similar to that of prostacyclin ($10^{5.1}$ mol/l), prostaglandin E_1 ($10^{-6.7}$ mol/l), and prostaglandin $F_{2\alpha}$ ($10^{-5.8}$ mol/l).

From calculations of food consumption, rats receiving food with concentrations of 0.03% and 0.1% cilostazol receive the equivalent of 58.6 and 195.9 mg $\cdot$ kg $^{-1}$ $\cdot$ day $^{-1}$. Determinations of plasma cilostazol

using the method of Akiyama et al. [7] on five normal rats fed 0.1% and two groups of five diabetic rats fed 0.03% and 0.1% cilostazol, respectively yielded values of 107 ± 12 , 370 ± 131 and 522 ± 151 ng/ml, respectively (Dr. Y. Kimura, Otsuka Pharmaceutical Co., Ltd, Tokushima, Japan; personal communication). These levels are well within the range found in human subjects fed a single 100 mg dose. Cilostazol in humans was well tolerated and was found to be rapidly absorbed, reaching peak plasma levels of 764 ng/ml at 3 h with an elimination half-life of 2.2 h (α -phase) and 18.0 h (β -phase) [7].

Cilostazol could improve peripheral nerve function by means of several mechanisms. First, conduction slowing has been ascribed to reduced nerve Na,K-ATPase activity [16]. Cilostazol has been shown to normalize this deficiency in STZ-diabetic nerves [17]. Cilostazol did not improve blood glucose levels; thus its beneficial effects are clearly unrelated to correction of hyperglycaemia. Shindo et al. [17] has previously reported electrophysiologic improvement without any correction of blood glucose levels or of *myo*-inositol in EDN. Our study is the first to demonstrate that cilostazol has a direct effect on NBF, suggesting that an alternative or additional mechanism for improving nerve electrophysiology might be through improvement in endoneurial perfusion. A second action relevant to diabetes is its potent inhibition of cGMP-inhibited, cAMP-selected phosphodiesterase (type III) [8] with an (IC_{50}) of 0.19 μ mol/l. A third action, of particular relevance to the diabetic peripheral nerve, is its ability to lower thromboxane B_2 without altering 6-keto prostaglandin $F_{1\alpha}$, the stable metabolite of prostacyclin. There was restoration of the ratio toward normal both in an experimental preparation [18] and in human subjects [19]. The thromboxane B_2 : prostacyclin ratio is increased in EDN [20]. The demonstration that oral administration will improve the NBF alterations and electrophysiologic abnormalities in EDN are especially important in suggesting that ingestion of the drug is potentially efficacious in diabetic neuropathy. Controlled clinical studies in human diabetic neuropathy are needed. EDN is very important in creating a model for early neuropathic alterations, but the chronic progressive diabetic changes in human nerve tissue probably involves additional mechanisms.

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References

1. Tuck RR, Schmelzer JD, Low PA (1984) Endoneurial blood flow and oxygen tension in the sciatic nerves of rats with experimental diabetic neuropathy. *Brain* 107: 935–950

2. Yao JK, Low PA (1986) Improvement of endoneurial lipid abnormalities in experimental diabetic neuropathy by oxygen modification. *Brain Res* 362: 362–365
3. Low PA, Schmelzer JD, Ward KK, Curran GL, Poduslo JF (1988) Effect of hyperbaric oxygenation on normal and chronic streptozotocin diabetic peripheral nerves. *Exp Neurol* 99: 201–212
4. Low PA, Tuck RR, Dyck PJ, Schmelzer JD, Yao JK (1984) Prevention of some electrophysiologic and biochemical abnormalities with oxygen supplementation in experimental diabetic neuropathy. *Proc Natl Acad Sci U S A* 81: 6894–6898
5. Cameron NE, Cotter MA, Low PA (1991) Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. *Am J Physiol* 261: E1–E8
6. Sonobe M, Yasuda H, Hisanaga T et al. (1991) Amelioration of nerve Na(+)-K(+)-ATPase activity independently of myo-inositol level by PGE1 analogue OP-1206.alpha-CD in streptozotocin-induced diabetic rats. *Diabetes* 40: 726–730
7. Akiyama H, Kudo S, Odomi M, Shimizu T (1985) High-performance liquid chromatographic procedure for the determination of a new antithrombotic and vasodilating agent, cilostazol, in human plasma. *J Chromatogr* 338: 456–459
8. Okuda Y, Kimura Y, Yamashita K (1993) Cilostazol. *Cardiovasc Drug Rev* 11: 451–465
9. Kawamura K, Watanabe K, Kimura Y (1985) Effect of cilostazol, a new antithrombotic drug, on cerebral circulation. *Arzneimittelforschung* 35: 1149–1154
10. Kihara M, Low PA (1990) Regulation of rat nerve blood flow: role of epineurial alpha-receptors. *J Physiol* 422: 145–152
11. Marquardt DW (1963) An algorithm for least-squares estimation of nonlinear parameters. *J Soc Indust Appl Math* 11: 431–441
12. Low PA, Schmelzer JD (1983) Peripheral nerve conduction studies in galactose-poisoned rats. Demonstration of increased resistance to ischemic conduction associated with endoneurial edema due to sugar alcohol accumulation. *J Neurol Sci* 59: 415–421
13. Low PA, Ward K, Schmelzer JD, Brimijoin S (1985) Ischemic conduction failure and energy metabolism in experimental diabetic neuropathy. *Am J Physiol* 248: E457–E462
14. Zochodne DW, Huang ZX, Ward KK, Low PA (1990) Guanethidine-induced adrenergic sympathectomy augments endoneurial perfusion and lowers endoneurial microvascular resistance. *Brain Res* 519: 112–117
15. Zochodne DW, Ho LT (1991) Influence of perivascular peptides on endoneurial blood flow and microvascular resistance in the sciatic nerve of the rat. *J Physiol* 444: 615–630
16. Greene DA, Lattimer SA (1983) Impaired rat sciatic nerve sodium-potassium adenosine triphosphatase in acute streptozotocin diabetes and its correction by dietary myo-inositol supplementation. *J Clin Invest* 72: 1058–1063
17. Shindo H, Tawata M, Onaya T (1993) Cyclic adenosine 3',5'-monophosphate enhances sodium, potassium-adenosine triphosphatase activity in the sciatic nerve of streptozotocin-induced diabetic rats. *Endocrinology* 132: 510–516
18. Igawa T, Tani T, Chijiwa T et al. (1990) Potentiation of antiplatelet aggregating activity of cilostazol with vascular endothelial cells. *Thrombosis* 57: 617–633
19. Nakagawa Y, Onuki Y, Orino H (1986) Effect of cilostazol (10P-13013) on arachidonic acid metabolism. *Jpn Pharmacol Ther* 14: 6319–6324
20. Ward KK, Low PA, Schmelzer JD, Zochodne DW (1989) Prostacyclin and noradrenaline in peripheral nerve of chronic experimental diabetes in rats. *Brain* 112: 197–208