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Cilostazol prevents impairment of slow axonal transport in streptozotocin-diabetic rats

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Abstract

We studied the effects of cilostazol, an antiplatelet and vasodilating agent, on axonal transport patterns of cytoskeletal proteins in the motor fibers of sciatic nerve of streptozotocin-induced diabetic rats. Proteins labeled with L-[^{35}S]methionine in 6-mm consecutive segments of the nerve were analyzed electrophoretically following fractionation into Triton-soluble and-insoluble subpopulations. Transport rates of proteins (particularly neurofilaments) in slow component a were reduced by 50% 2 weeks after labeling (4 weeks after streptozotocin). An apparent reduction of tubulin and actin was observed at later intervals after induction of diabetes. Actin transported in slow component b was also impaired, though to a lesser extent than in component a. Cilostazol prevented transport impairment of both slow components a and b without affecting hyperglycemia or reduction in body weight gain. These results suggest that in sciatic motor fibers early defects in slowly transported proteins are more marked in slow component a, and that impairment may be caused primarily by hemodynamic abnormalities.

Keywords: Streptozotocin-diabetic, rat; Cilostazol; Antiplatelet vasodilating agent; Phosphodiesterase inhibitor

1. Introduction

Diabetic neuropathy is one of the most frequent complications seen in diabetic patients. A previous study by Sima et al. (1988a) reported that axonal atrophy is characteristic in patients with type I insulin-dependent diabetes mellitus. Additionally, streptozotocin-induced diabetic rats (an animal model of type I) also display axonal atrophy in both sensory and motor fibers of sciatic nerves (Sima et al., 1988b; Yagihashi et al., 1990). Axonal atrophy is defined by the amount of cytoskeletal proteins such as neurofilaments, tubulins and actin, in particular the number of neurofilaments. In the nerve, neurofilaments, tubulins and actin are transported down the axon by the slow axonal transport system. In genetically or experimentally induced diabetic models, impairment of slow axonal transport has been widely reported (Sidenius and Jakobsen, 1982; Mayer et al., 1984; Medori et al., 1985, 1988; Vitadello et al., 1985; Macioce et al., 1989). This impairment, which has often developed as a primary event in early diabetic neuropathy, causes axonal atrophy that eventually leads to a decrease in nerve conduction velocity (Tomlinson and Mayer, 1984; Larsen and Sidenius, 1989). Thus, the major cause for diabetic neuropathy can be attributed to an abnormality of slow axonal transport. Slow axonal transport of cytoskeletal proteins can be divided into two subcomponents, which differ in transport rate and composition (Black and Lasek, 1980; Levine and Willard, 1980; Tytell et al., 1981). Thus, for the present study we generated two subcomponents of slow axonal transport, slow component a and b, which can be clearly distinguished in sciatic motor fibers by solubilizing proteins in the presence of 1% Triton X-100 at 4°C (Tashiro et al., 1984; Tashiro and Komiya, 1989).

Impaired axonal transport can be improved by insulin treatment, implying involvement of metabolic factors (Larsen and Sidenius, 1989). In addition to metabolic factors, hemodynamic abnormalities of peripheral nerves, such as nerve hypoxia or ischemia, are believed to be major causes of functional deterioration in diabetic neuropathy of strep-

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tozotocin-diabetic rats. Vasodilators such as prostaglandin E_1 analogues or α-adrenergic receptor blockers ameliorate the reduced motor nerve conduction velocity and Na^+,K^+-ATPase activity (Sonobe et al., 1991; Cameron et al., 1991). However, there are no reports concerning the effects of vasoactive agents on the impaired slow axonal transport in diabetic rats.

Recent studies have shown that cilostazol, an antiplateled agent with vasodilating action (Tanaka et al., 1988; Tani et al., 1992), increased endoneurial blood flow (Kihara et al., 1995b) and improved delayed nerve conduction velocity, decreased nerve Na^+,K^+-ATPase activity and axonal atrophy in streptozotocin-diabetic rats (Shindo et al., 1993; Naka et al., 1995; Sasaki et al., 1997; Uehara et al., 1997). We have previously reported that cilostazol markedly prevented a decrease in axonal regeneration rates following nerve injury in streptozotocin-induced diabetic rats (Yamamoto et al., 1998). Therefore, for this study we examined the actions of cilostazol on slow axonal transport in short-term diabetes. Our results showed that cilostazol prevented the impaired slow axonal transport without affecting metabolic factors, suggesting that hemodynamic abnormalities may play a part in impairment of slow axonal transport.

2. Materials and methods

2.1. Animals

Male Wistar rats (120–160 g, Nihon SLC, Japan) were used at 6 weeks of age. The animals were housed in a controlled environment (23 ± 2°C, 60 ± 10% relative humidity) and allowed free access to food and water. The room lights were turned off between 1900 and 0700 h. All experimental procedures were carried out in accordance with the guidelines and instructions for animal experimentation recommended by the Science Council of Japan.

2.2. Induction of diabetes

Diabetes was induced in ether-anesthetized animals by an intravenous injection of streptozotocin (Wako, Japan) at a dose of 50 mg/kg. Normal (control) rats received vehicle (0.01 M citrate-saline buffer, pH 4.5) alone.

2.3. Experimental groups and administration of cilostazol

Three days after injection of streptozotocin, plasma glucose concentrations were measured using the glucose oxidase method (Glucose B-test Wako, Wako), and rats with non-fasting blood glucose > 400 mg/dl were considered to be diabetic. Body weight and plasma glucose levels were monitored for all experimental rats before streptozotocin injection, at 3 days and 2 weeks post-injection, and at the end of the experiment. Animals identified as diabetic were randomly divided into two groups: untreated diabetic ($n = 9$) and cilostazol-treated diabetic ($n = 9$). Cilostazol-treated rats were given a pelleted diet containing 0.03% cilostazol with free access to water and chow. Both untreated diabetic rats and age-matched normal rats (non-diabetic control group, $n = 9$) were given standard rat chow.

2.4. Radioactive labeling and transport of cytoskeletal proteins

Fourteen days after streptozotocin injection, L-[^{35}S]-methionine (185 MBq; New England Nuclear, North Bellerica, MA, USA), concentrated by lyophilization (0.82 MBq in 0.2 μl), was injected into the anterior horn area of the L_4–L_5 spinal cord twice on each side. At one, 2 or 3 weeks after isotope injection, sciatic nerves with ventral roots (L_4 and L_5) were dissected out from three animals in each group, frozen on a plastic plate, and cut into 6-mm consecutive segments. Each 6-mm segment was dissolved in 500 μl of sodium dodecyl sulfate sample buffer (2% SDS, 80 mM Tris, pH 6.8, 5 mM mercaptoethanol, and 10% glycerol) and subjected to gel electrophoresis on 10% acrylamide gels. Labeled proteins on the gel were visualized by fluorography.

2.5. Fractionation of labeled cytoskeletal proteins

Sciatic nerves obtained at each time point after labeling were subjected to further fractionation of transported proteins (six animals per group). A pair of nerve segments was frozen in liquid nitrogen, crushed to a fine powder, and homogenized in 2.5 ml of ice-cold Triton buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 25 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 5 mM dithiothreitol, and 0.25 mM phenylmethylsulfonylfluoride. The homogenate was layered onto 2 ml of Triton buffer containing 0.25 M sucrose and centrifuged at $100{,}000 \times g$ for 1 h. Triton-soluble proteins in the resulting supernatant were precipitated with cold 10% (final concentration) trichloroacetic acid, washed with ethanol, and dissolved in 500 μl of SDS sample buffer. Triton-insoluble proteins in the precipitate were directly dissolved in 500 μl of SDS sample buffer. Labeled proteins in both the soluble and insoluble fractions were separated by SDS-gel electrophoresis. Radioactivity associated with bands of NF160, NF68 (160-, 68-kDa neurofilament subunits, respectively), α + β tubulins and actin was directly quantified after the dried gels were exposed for 2 days to a radiation-sensitive Imaging Plate (Fuji Film, Tokyo, Japan) and visualized on a BAS model 2000 Bioimage Analyzer (Fuji Film).

2.6. The peak position of transported cytoskeletal proteins

Distribution of radioactivity of each cytoskeletal protein along the sciatic nerve was normalized as a percentage of total radioactivities in all 15 nerve segments. Location of

peak of radioactivity in slow component a was determined from the distribution pattern of the Triton-insoluble subfraction, and that of slow component b from the Triton-soluble subfraction.

2.7. Statistical analysis

Data were analyzed by analysis of variance and multiple comparisons testing (Tukey's method) using Statistical Analysis System (Cary, NC, USA). Results were expressed as mean ± S.E.M. Differences with a $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cilostazol does not affect on body weight and plasma glucose levels

Body weight in untreated diabetic and cilostazol-treated diabetic rats was markedly reduced compared with normal control rats; values at the end of study in normal, untreated diabetic and cilostazol-treated diabetic rats were 270 ± 3, 170 ± 16 and 177 ± 13 g, respectively (data not shown). Plasma glucose level in untreated diabetic and cilostazol-treated diabetic rats showed persistent hyperglycemia; values at the end of study were 718 ± 32 and 647 ± 55 mg/dl, respectively (data not shown). There were no differences between untreated diabetic and cilostazol-treated diabetic rats in body weight and plasma glucose levels and cilostazol had no effect on these aspects of the diabetic state during the experimental period.

3.2. Cilostazol prevents the impairment of slow axonal transport in diabetic rats

Fig. 1 shows fluorograms obtained from the sciatic nerve of normal control, untreated diabetic and cilostazol-treated diabetic rat 3 weeks after the isotope injection into the spinal cord. Radioactivity associated with cytoskeletal proteins was distributed within the more proximal segments of the nerve in untreated diabetic rat compared to normal control rat (Fig. 1A,B), suggesting a significant reduction in the slow axonal transport rate due to diabetes. Retardation was especially prominent with NF160 and NF68. Compared with NF68, the 70-kDa band with higher rate of transport (marked with a asterisks in (Fig. 1A–C) consists of two different classes of proteins HSC70 (heat shock protein) and annexin VI, which are unrelated to NF68.

Administration of cilostazol to diabetic rat resulted in a transport pattern apparently indistinguishable from that of normal control rat (Fig. 1C). Thus, cilostazol showed a protective effect on the impairment of slow axonal transport in diabetic rat.

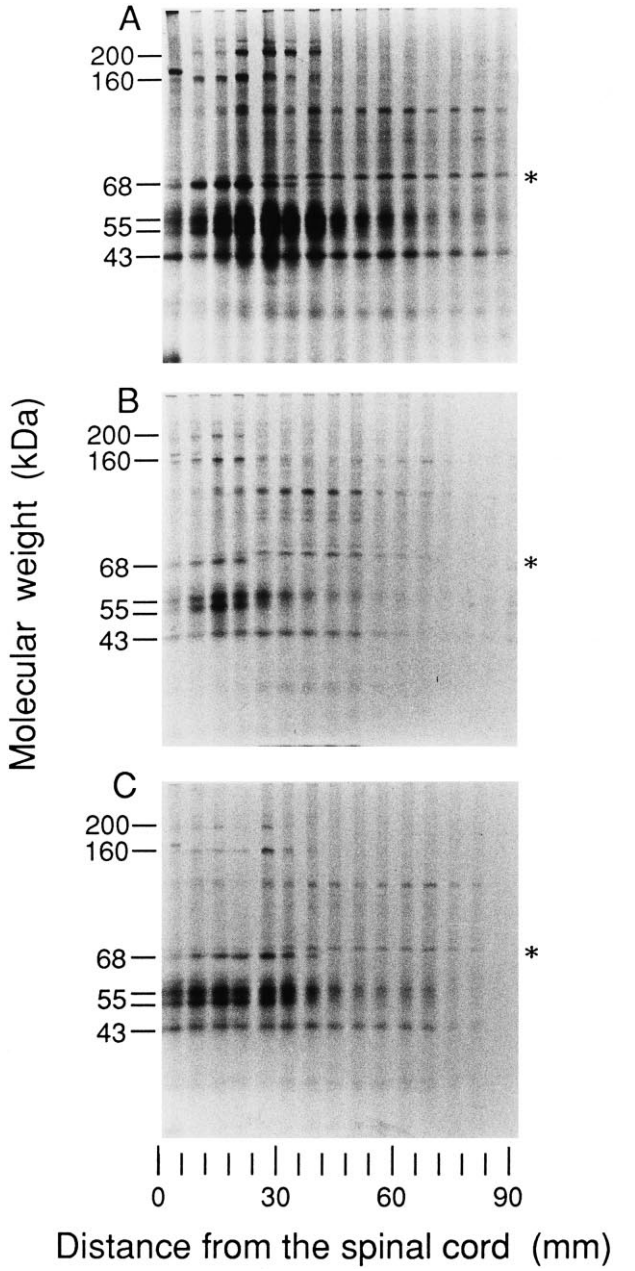

Fig. 1. Effect of diabetes on slowly transported proteins in the sciatic motor nerve. Three weeks after injection of L-[^{35}S]methionine into the spinal cord, labeled proteins in consecutive 6-mm segments of the ventral root and the sciatic nerve were separated by SDS-gel electrophoresis and detected by fluorography. Compared to normal control rat (A), there was a significant reduction in slow axonal transport rate in untreated diabetic control rat (B). Treatment of diabetic rat with cilostazol (C) resulted in a normal transport pattern. Neurofilament subunits (200,000, 160,000 and 68,000 molecular weight), tubulin (55,000) and actin (43,000) are indicated in the figure. * = HSC70 and annexin VI proteins.

3.3. Cilostazol affects the axonal transport of individual cytoskeletal components

In order to analyze the effects of diabetes and cilostazol treatment on axonal transport of the three major cytoskeletal components individually, we further fractionated

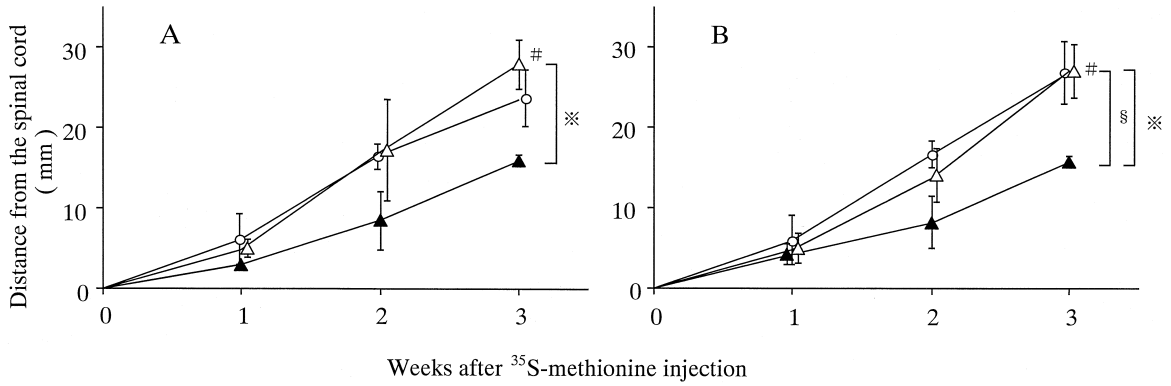

Fig. 2. Comparison of transport patterns of neurofilament proteins in slow component a among normal control (○), untreated diabetic (▲) and cilostazol-treated diabetic group (△). Two weeks after induction of diabetes, L-[^{35}S]methionine was injected into the spinal cord and sciatic nerves obtained at each time point were cut into consecutive 6-mm segments. A pair of nerve segments was fractionated into Triton-insoluble subpopulations and separated by SDS-gel electrophoresis. From the radioactivity profile of each band, migrated distance of labeled proteins was calculated. Transport rates of NF160 (A) and NF68 (B) were decreased in untreated diabetic rates, compared to normal control and cilostazol-treated diabetic rats. Each point represents mean ± S.E.M. ($N = 3–4$). $^{\#}P < 0.05$: Against corresponding values of untreated diabetic group. $^{\S}P < 0.05$: Between normal control and untreated diabetic group. $^{※}P < 0.05$: Between untreated and cilostazol-treated diabetic group.

the labeled proteins into slow component a (Triton-insoluble) and b (Triton-soluble) subcomponents. Using the Triton-insoluble fraction, positions of the peaks of radioactivity associated with NF160 and NF68 in diabetic rats were located at different time intervals after radioactive labeling compared to normal rats. Results presented in Fig. 2 clearly indicate that the advancement of the radioactive peaks was retarded in untreated diabetic rats. Decreases in transport rates of NF160 and NF68 in untreated diabetic, compared to normal control, rats were 49% and 51%, respectively, 2 weeks after labeling (4 weeks of diabetes). Treatment of diabetic rats with cilostazol prevented the impairment of neurofilament transport and maintained the rates at normal levels (Fig. 2A,B).

In contrast to neurofilaments, which are confined to slow component a, tubulin and actin are present both in slow components a and b. Radioactive peaks of slow component a tubulin and actin were determined using the Triton-insoluble fraction, while those of slow component b tubulin and actin were determined using the Triton-soluble fraction, which gives a clear biphasic pattern of transport.

A decrease in transport rates of tubulin (39%) and actin (44%) in slow component a was observed at 3 weeks post-labeling (5 weeks of diabetes) (Fig. 3). However, the onset of retardation was found significantly later in cases of tubulin and actin as the rates of advancement of the peaks were indistinguishable from those of normal control rats up to 2 weeks after labeling (4 weeks of diabetes). As

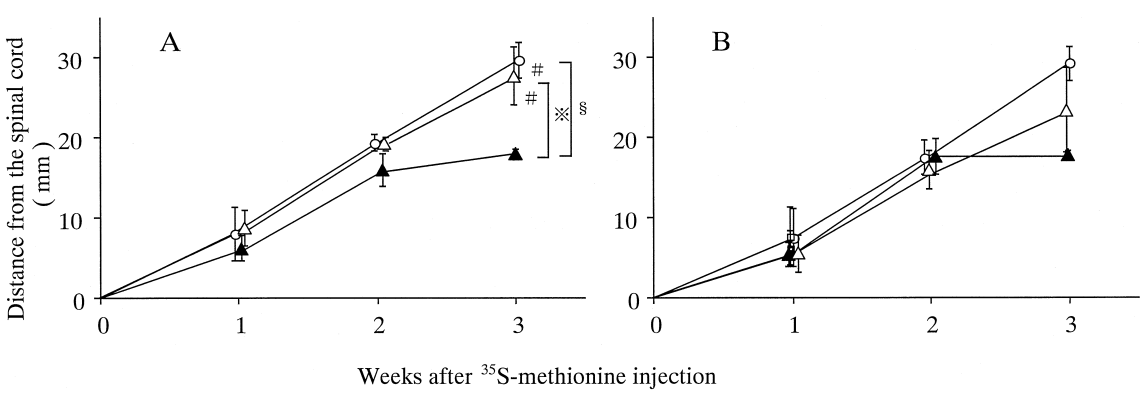

Fig. 3. Comparison of transport patterns of tubulin and actin in slow component a among normal control (○), untreated diabetic (▲) and cilostazol-treated diabetic group (△). Two weeks after induction of diabetes, L-[^{35}S]methionine was injected into the spinal cord and sciatic nerves obtained at each time point were cut into consecutive 6-mm segments. A pair of nerve segments was fractionated into Triton-insoluble subpopulations and separated by SDS-gel electrophoresis. From the radioactivity profile of each band, migrated distance of labeled proteins was calculated. Transport rates of tubulin (A) and actin (B) were decreased in untreated diabetic rates, compared to normal control and cilostazol-treated diabetic rats. Each point represents the mean ± S.E.M. ($N = 3–4$). $^{\#}P < 0.05$: Against corresponding values of untreated diabetic group. $^{\S}P < 0.05$: Between normal control and untreated diabetic group. $^{※}P < 0.05$: Between untreated and cilostazol-treated diabetic group.

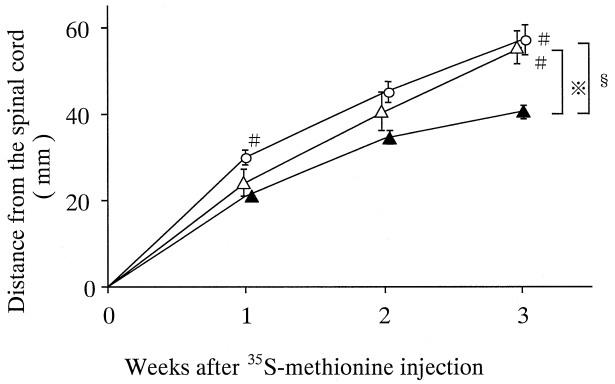

Fig. 4. Comparison of transport pattern of actin in slow component b among normal control (⊄), untreated diabetic (″) and cilostazol-treated diabetic group (≥). Two weeks after induction of diabetes, L-[^{35}S]methionine was injected into the spinal cord. The sciatic nerves obtained at each time point were cut into consecutive 6-mm segments and a pair of nerve segments were fractionated into Triton-soluble subpopulations and separated by SDS-gel electrophoresis. From the band radioactivity profiles, migrated distance of labeled proteins was calculated. Transport rate of actin, but not tubulin, was decreased in untreated diabetic rats. This retardation of slow component b actin was prevented by treatment with cilostazol. Each point represents mean ± S.E.M. ($N = 3$–4). $^{\#}P < 0.05$: Against corresponding values of untreated diabetic group. $^{\S}P < 0.05$: Between normal control and untreated diabetic group. $^{*}P < 0.05$: Between untreated and cilostazol-treated diabetic group.

in the case of neurofilament transport, treatment of diabetic rats with cilostazol also prevented the transport impairment of tubulin and actin carried in slow component a.

Transport rate of soluble actin, which is a major component of slow component b, was also decreased in diabetic rats (Fig. 4). The extent of the rate reduction was smaller compared with that of the proteins carried in slow component a (29% at 3 weeks post-labeling). Retardation was detectable at the earliest time interval analyzed (1 week after labeling) as was the case of neurofilaments in slow component a. In regard to tubulin carried in slow component b, we could not detect any differences in transport rates between untreated diabetic and normal control rats (data not shown), but the retardation of slow component b actin transport was prevented by cilostazol (Fig. 4).

4. Discussion

Previous studies of slow axonal transport in motor fibers have been controversial since it has been reported that transport of slow component b is more impaired than slow component a (Medori et al., 1985, 1988) or that neither slow component a nor b is impaired (Macioce et al., 1989). One of the possible reasons for such a discrepancy is that, due to a relatively smaller slow component b, these two components are not as readily distinguishable from each other in motor fibers as they are in the sensory fibers. For this study, by subfractionating the transported proteins into Triton-soluble (component b) and -insoluble (component a) subpopulations, we could obtain clear transport profiles of slow components a and b in motor fibers, which enabled us to clearly determine their individual transport rates.

Our results showed that in slow component a transport of neurofilaments was most severely impaired even at the early stage of experimental diabetes, while reduction in the rates of tubulin and actin occurred at least 2 weeks later than that of neurofilaments. These results suggest that impairment of neurofilament transport may trigger a defect in transport of other cytoskeletal proteins carried in slow component a. We have previously demonstrated that microtubule stabilization in the axon, which takes place mainly in the proximal region, is brought about at least in part through interaction with neurofilaments (Tashiro and Komiya, 1989). Therefore, the depletion of neurofilaments may alter the interaction between neurofilaments and other cytoskeletal components eventually leading to a defect in the transport of other cytoskeletal proteins. However, his does not clarify why the transport of neurofilaments would be impaired prior to that of any other cytoskeletal proteins.

Among factors possibly contributing to slow axonal transport impairment in the sciatic nerve of diabetic rats, it has been suggested that metabolic derangements including excessive non-enzymatic glycation of proteins and/or disruption of the polyol pathway arising from hyperglycemia may be involved (Vitadello et al., 1985; Willars et al., 1986; McLean et al., 1992). However, cilostazol prevented all of transport impairments without ameliorating the persistent hyperglycemia in this study, and it had no effect on glycated hemoglobin levels and sorbitol accumulation in sciatic nerve or in red blood cells in streptozotocin-diabetic rats (Uehara et al., 1997). These results suggest that metabolic derangement is not directly responsible for the transport impairment seen in short-term diabetes. This is supported by results showing that treatment with the aldose reductase inhibitor ponalrestat, which inhibits metabolic derangement occurring by activation of the polyol pathway, had no effect on the defect at 4–5 weeks in streptozotocin-diabetic rats (Mayer et al., 1984; Tomlinson et al., 1986). On the other hand, it has also been reported that aldose reductase inhibitors improve nerve blood flow in addition to metabolic derangement (Cameron et al., 1994, 1996). As mentioned above, however, ponalrestat did not ameliorate the impaired slow axonal transport (Mayer et al., 1984; Tomlinson et al., 1986). This inconsistency can be explained by difference in the given dose. Namely, although the efficacy of ponalrestat for increasing sciatic nerve blood flow is confirmed at 50 mg/kg or more (Calcutt et al., 1994; Cameron et al., 1994), the dose used in that experiment was 25 mg/kg and nerve blood flow was not measured. Therefore, it remains unclear whether at the dose used ponalrestat increased nerve blood flow.

In addition to metabolic derangement, there is growing evidence supporting the involvement of hemodynamic ab-

normalities, including nerve hypoxia or ischemia, in the pathogenesis of human diabetic neuropathy (Dyck et al., 1985, 1986; Powell et al., 1985; Sima et al., 1988a). In the sciatic nerve of streptozotocin-diabetic rats, the observed reduced nerve blood flow was attributed to nerve hypoxia, correlating with functional deficit of the nerve (Tuck et al., 1984; Low et al., 1984, 1988, 1989; Cameron, et al., 1991; Kihara and Low, 1995). Cilostazol improved the reduced endoneurial nerve blood flow, decreased nerve conduction velocity, and prevented morphological changes in myelinated fibers in sciatic nerves of streptozotocin-diabetic rats (Shindo et al., 1993; Kihara et al., 1995; Naka et al., 1995; Sasaki et al., 1997; Uehara et al., 1997). These effects of cilostazol are not due to a direct effect on the neuron because there is little type III phosphodiesterase activity in the anterior horn of spinal cord, where cell bodies of motor fibers reside (data not shown).

We conclude that the impairment of slow axonal transport in short-term diabetes may be caused primarily by hemodynamic abnormalities rather than metabolic derangement, and that the improvement observed with cilostazol could be explained by prevention of hemodynamic abnormalities. Further studies are necessary to clarify the precise mechanisms by which cilostazol ameliorates impaired slow axonal transport by elucidating the effects of cilostazol on endoneurial microangiopathy.

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