Effects of Beraprost Sodium, a Prostacyclin Analogue, on Diabetic Neuropathy in Streptozotocin-Induced Diabetic Rats

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ABSTRACT—The effects of beraprost sodium (BPS), a stable prostacyclin analogue, on motor nerve conduction velocity and nerve blood flow of the sciatic nerve were investigated in streptozotocin-induced diabetic rats, and they were compared with the effects of epalrestat (aldose reductase inhibitor). Treatment with BPS for 4 weeks significantly inhibited the decrease in motor nerve conduction velocity and nerve blood flow dose-dependently, but epalrestat had no effect on nerve blood flow. Morphological changes of the myelinated fibers of the sciatic nerve were observed macroscopically. The mean axonal area and the mean circularity index of diabetic control rats were significantly less than that of normal rats, while after 6 weeks of BPS treatment, these decreases of the axonal area and the circularity index were inhibited. The enlargement of the mean lumen area of microvessels in the diabetic rats was significantly inhibited after 6 weeks of BPS treatment. Additionally, augmentation of the washed platelet aggregation in diabetic rats was significantly normalized by BPS. It was suggested that BPS is effective on diabetic neuropathy via amelioration of the decrease of blood supply to the structure. The effects of BPS on platelets might also contribute to the improvement of neuronal circulatory deficiency.

Keywords: Beraprost sodium, Motor nerve conduction velocity, Nerve blood flow, Morphological analysis

There has been increasing evidence suggesting a vascular involvement in the pathogenesis of diabetic neuropathy (1-6). In experimental diabetic rats, reduction in nerve blood flow and decrease in endoneurial oxygen tension in the sciatic nerve have been attributed to ischemia in the endoneurium (1). Electrophysiological and biochemical abnormalities were improved with oxygen supplementation in these animals (2). Furthermore, involvement of the ischemic process in the development of neuropathy has been demonstrated by morphological studies with biopsied human nerve samples (3, 5). On the other hand, it is well-known that prostacyclin has a potent inhibitory effect on platelet aggregation and vasodilatory effects (7, 8). However, its rapid metabolic degradation in vivo has limited its therapeutic application. Beraprost sodium (sodium ((±)-1R*,2R*,3aS*,8bS*)-2,3,3a,8b-tetrahydro-2-hydroxyl-1H-cyclopenta[b]benzofuran-5-butyrate; BPS) is a chemically stable prostacyclin analogue that can be administered orally and has been demonstrated to possess a similar pharmacological profile to prostacyclin; i.e., potent antiplatelet aggregation effect in vitro and ex vivo in various animal species (9, 10), antithrombotic effect on arterioles in the hamster cheek pouch (11), antithromboembolic effect in mice (12) and vasodilating effects in the dog (13). Thus, in view of the abnormalities of the microcirculation in the pathogenesis of diabetic neuropathy, the pharmacological profile of BPS might suggest its possible effectiveness in diabetic neuropathy.

In this report, we examined whether BPS prevents diabetic neuropathy in streptozotocin-induced diabetic rats by its vasodilatory and antiplatelet aggregation effect. In addition, we investigated whether improvement of the microcirculation contributes to the prevention of development of diabetic neuropathy.

MATERIALS AND METHODS

Induction of diabetes and experimental protocol
Diabetes was induced by a single i.p. injection of streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO, USA) at a dose of 55 mg/kg in male Sprague-Dawley rats (225-260 g; SLC, Shizuoka). Two sets of experiments were serially conducted (Fig. 1). The first experiment was designed to study the effect of BPS on motor nerve conduction velocity (MNCV) and nerve...
blood flow (NBF) of the sciatic nerve. In the second experiment, morphological analysis and platelet aggregation in diabetic rats were performed.

**Experiment 1:** Eight weeks after the induction of diabetes, plasma glucose levels were measured, and diabetic animals were divided into 4 groups. STZ diabetic rats without treatment were used as the control (n=5). BPS treated groups (10 µg/kg/day, n=5; 30 µg/kg/day, n=4) and the epalrestat-treated group (50 mg/kg/day, n=6) were studied after 4 weeks of treatment. Untreated, age-matched rats (n=5) served as the normal group. After the measurement of plasma glucose, diabetic and normal rats were weighed, and anesthetized. The rectal temperature was maintained at 36±0.5°C with a heating pad. The left sciatic nerve was stimulated at the sciatic notch and the tibial nerve at the ankle by bipolar electrodes at supramaximal stimuli. Distal and proximal latencies were measured from oscilloscope recordings, and the velocity was calculated. NBF was measured with the laser Doppler flowmetry technique (ALF 2100; Advance Co., Ltd., Tokyo). The probe was applied vertically to the sciatic nerve with least pressure until the measurement become stable. Although the laser Doppler flowmetry technique does not always provide an absolute value of the blood flow, this technique was thought to be the best suited method for this study. For comparison of different groups, the absolute value is not significant, although some calibration might be inevitable.

**Experiment 2:** Twelve weeks after the injection of STZ, plasma glucose levels were measured, and diabetic animals were divided into two groups. They were kept on various treatments for a further 6 weeks. At 18 weeks after the induction of diabetes, the neurological examinations were performed on rats with untreated diabetes, the control group (n=5), BPS-treated group (30 µg/kg/day, n=5) and age-matched normal group (n=4). Under diethyl ether anesthesia, blood was removed from the carotid artery and collected into tubes containing 3.8% trisodium citrate dihydrate. Washed platelets were prepared from diabetic rats and age-matched normal rats. Platelet maximum aggregation was measured by the turbidimetric method with ADP (10 µM) at 37°C with constant stirring at 1000 rpm. After blood sample collection for platelet aggregation, the left side of the sciatic nerve was removed and fixed in 2% glutaraldehyde in 0.025 M cacodylate buffer, pH 7.38, at 4°C for 2 hr. Tissue blocks were further fixed in osmium tetroxide in buffer at room temperature and embedded in epoxy resin. Transverse semithin sections (1 µm) were stained with toluidine blue. The nerve fascicle and the microvessels were randomly selected for measurements of the mean myelinated nerve fibers area, the mean circularity index and the mean lumen area. Morphometric analyses were performed to calculate the average in the focal lesion of slides containing for each of the three sites by means of a computer-assisted image analyzing system (SP 500; Olympus, Tokyo). The mean myelinated nerve fibers area, the mean circularity index and the mean lumen area of microvessels were calculated by computer.

**Drugs and statistical analyses**

BPS, prepared at the Chemical Laboratory of the Basic

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**Fig. 1.** Experimental protocol. Experiment 1: Beraprost sodium (BPS) administration for 4 weeks was started 8 weeks after streptozotocin (STZ) injection. Experiment 2: BPS administration for 6 weeks was started 12 weeks after STZ injection. ARI, aldose reductase inhibitor (epalrestat); MNCV, motor nerve conduction velocity; NBF, nerve blood flow; Aggregation, platelet aggregation.
Research Laboratories in Toray Industries, Inc., was dissolved in distilled water to make a series of test solutions. Epalrestat (aldose reductase inhibitor), prepared at the Toray Research Center in Toray Industries, Inc., was suspended in 1% CMC solution. The data are expressed as means ± S.D. of 4 to 6 experiments. Statistical analyses were performed by the unpaired Student’s t-test. Differences among diabetic groups in Experiment 1 were detected by analysis of variance (ANOVA), and significance at the 0.05 level was assessed by Dunnett’s test.

RESULTS

Experiment 1

Weight gain of the diabetic groups was less than normal. All rats in the diabetic groups showed persistent hyperglycemia during the experiment, and neither BPS nor epalrestat treatment had any effect on plasma glucose level (Table 1). At 12 weeks, MNCV was significantly lower in diabetic control rats (20.59 ± 2.57 m/s) than in normal rats (42.76 ± 11.45 m/s). BPS significantly in-

| Experiment 1 | Pre (8 weeks) | Post (12 weeks) |
|--------------|---------------|-----------------|
| Group/treatment | n | body weight (g) | plasma glucose (mg/dl) | body weight (g) | plasma glucose (mg/dl) |
| Normal | 5 | 420 ± 25 | 162.2 ± 23.1 | 458 ± 26 | 164.2 ± 27.9 |
| Diabetic | | | | |
| Control | 5 | 215 ± 25 | 554.8 ± 30.4 | 211 ± 21 | 585.6 ± 46.3 |
| BPS (10 μg/kg) | 5 | 220 ± 30 | 533.2 ± 67.6 | 215 ± 33 | 597.2 ± 36.6 |
| BPS (30 μg/kg) | 4 | 216 ± 15 | 568.8 ± 69.2 | 201 ± 19 | 642.5 ± 43.7 |
| ARI (50 mg/kg) | 6 | 224 ± 27 | 593.3 ± 103.1 | 199 ± 25 | 598.0 ± 8.5 |

| Experiment 2 | Pre (12 weeks) | Post (18 weeks) |
|--------------|---------------|-----------------|
| Group/treatment | n | body weight (g) | plasma glucose (mg/dl) | body weight (g) | plasma glucose (mg/dl) |
| Normal | 5 | 484 ± 45 | 156.4 ± 36.0 | 523 ± 58 | 159.4 ± 20.9 |
| Diabetic | | | | |
| Control | 6 | 183 ± 23 | 505.4 ± 49.2 | 189 ± 29 | 474.2 ± 25.0 |
| BPS (30 μg/kg) | 6 | 208 ± 13 | 478.2 ± 56.7 | 210 ± 22 | 508.3 ± 56.5 |

Values are means ± S.D. Experiment 1: Beraprost sodium (BPS) administration for 4 weeks started 8 weeks after streptozotocin (STZ) injection. Experiment 2: BPS administration for 6 weeks was started 12 weeks after STZ injection. ARI, aldose reductase inhibitor (epalrestat).

Fig. 2. Effect of beraprost sodium (BPS) administration on nerve conduction velocity (a) and nerve blood flow (b) in streptozotocin (STZ)-diabetic rats. Values are means ± S.D. of 4 to 6 rats. *P < 0.05, **P < 0.01 vs diabetic control group by the unpaired Student’s t-test. *P < 0.05, **P < 0.01 vs diabetic control group by Dunnett’s test. ARI, aldose reductase inhibitor (epalrestat). Open column, normal; hatched column, diabetics.
hibited the decrease of MNCV dose-dependently (Fig. 2a). Epalrestat treatment also significantly inhibited the MNCV decrease. No statistical difference was observed between treated groups. At 12 weeks, NBF of the sciatic nerve was significantly less in diabetic control rats (8.32 ± 0.83 ml/min/100 g) than in normal rats (17.68 ± 2.46 ml/min/100 g; Fig. 2b). After 4 weeks treatment of BPS, NBF was significantly improved, dose-dependently (Fig. 2b), but epalrestat (10.75 ± 3.35 ml/min/100 g) did not inhibit the decrease of NBF.

Experiment 2
All rats in the diabetic groups showed persistent hyperglycemia during the experiment, and BPS treatment did not have any effect on plasma glucose level (Table 1). The mean axonal area of diabetic control rats was significantly less than that of normal rats (diabetic, 40.73 ± 3.84; normal, 54.97 ± 5.62 μm²), and after 6 weeks treatment of BPS, this decrease of the axonal area was reversed (52.18 ± 6.62 μm²; Fig. 3a). The mean circularity index of diabetic control rats was significantly less than that of normal rats (diabetic, 81.09 ± 1.44; normal, 87.49 ± 2.79%). However, this reduction in circularity index was reversed to 85.06 ± 2.01% by the treatment with BPS (Fig. 3b). The mean lumen area of microvessel abnormality in the diabetic control rats was significantly improved after 6 weeks of BPS treatment (Fig. 3c).

ADP-induced aggregation was enhanced in washed platelets from diabetic control rats. This abnormal platelet function was significantly improved after 6 weeks of BPS treatment (Fig. 4).

DISCUSSION
In this study, neither BPS nor epalrestat treatment had any effect on plasma glucose level and body weight (Table 1). On the other hand, both BPS and epalrestat significantly inhibited the decrease of MNCV (Fig. 2). Therefore, the improvement of MNCV by BPS and epalrestat is not due to the secondary result of the hypoglycemic effect.

Several studies have demonstrated that prostacyclin may affect the development of diabetic neuropathy in both experimental animals and patients (14, 15). As shown in Fig. 2a, BPS, a stable prostacyclin analogue, significantly prevented the decrease in MNCV in diabetic rats. Thus, BPS might be useful as a therapeutic agent for diabetic neuropathy.

Involvement of the ischemic process in the development of neuropathy has been demonstrated by morphological studies with biopsied human nerve samples (3, 5). In the experimental diabetic rats, reduction in NBF and decrease in endoneurial oxygen tension in the sciatic nerve have been attributed to ischemia in the endoneurium (1). In the present study, sciatic NBF was significantly less in the diabetic control rats than in normal rats, and this decrease was dose-dependently reversed with 4 weeks
treatment of BPS (Fig. 2b). However, epalrestat did not inhibit the decrease of sciatic NBF. These data suggest that BPS might improve the microcirculation of nerves and significantly inhibited the decrease of MNCV (Fig. 2a) in the diabetic rats. Thus, we reconfirmed that the deficiency of the microcirculation might be related to the development of diabetic neuropathy. Moreover, the mechanism of the effectiveness of epalrestat on neuronal function might not be relevant to circulatory improvement.

In diabetic patients, plateslets were shown to exhibit increased sensitivity to aggregating agents (16–18). In this experiment, platelet aggregation was enhanced in STZ-induced diabetic rats. This abnormal platelet function is in accordance with the findings by Wada et al. (19) that aggregation is enhanced in washed plateslets from STZ-induced diabetic rats. The platelet aggregation abnormality in diabetic rats was significantly improved after 6 weeks treatment with BPS (Fig. 4). This result indicates that chronic treatment of BPS suppressed the increased sensitivity to aggregating agents in diabetic rats in the absence of BPS in blood concentration. In the diabetic state, rheological abnormality of the blood might cause a decrease of NBF (20). Hirano et al. (21) suggested that BPS lessens the hemorrhheological impairment in an animal model of peripheral circulation disorder. This may be due to decreases in the pathophysiologic changes that occur in the diabetic state which increases blood viscosity, fibrinogen concentration, platelet aggregation, and decreases erythrocyte deformability. By lessening these pathophysiologic changes, BPS might contribute to the amelioration of neuronal circulatory deficiency as well.

The morphometric data concerning the lumen area of endoneurial microvessels showed dilated arterial vessels in the diabetic control rats, and this dilatation was reversed by BPS treatment (Fig. 3c). Arteriolar and venular dilations in diabetic animals might be due to the dysfunction of vascular components (22, 23). These data suggest that BPS might improve the microcirculation of the nerve in diabetic rats. On the other hand, epalrestat did not reverse the dilation of the lumen area of endoneurial microvessels in the same model of diabetic rats (24). It was supposed that the mean axonal area and mean circularity index directly reflect the function of the nerve (25). The mean axonal area and mean circularity index of long term diabetic control rats were significantly less than that of normal rats. The decrease of these parameters were reversed by 6 weeks treatment of BPS (Fig. 3: a and b). The improvement of the circulation might have contributed to the morphological change of the nerve as well. These morphological data suggest that BPS alleviates axonal atrophy through a mechanism involving endoneurial microcirculatory improvement.

It was suggested from the above experiments that BPS is effective on diabetic neuropathy by augmentation of the decreased blood supply to the neuronal structure in diabetic condition.

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