Amelioration of Nerve Na^+-K^+-ATPase Activity Independently of *myo*-Inositol Level by PGE_1 Analogue OP-1206 · α-CD in Streptozocin-Induced Diabetic Rats

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An oral prostaglandin E_1 (PGE_1) analogue, OP-1206 · α-CD, was given to rats with streptozocin (STZ)-induced diabetes to examine the therapeutic effects of OP-1206 on short-term and long-term diabetic neuropathy and its action mechanism with special reference to nerve Na^+-K^+-ATPase activity. In the short-term experiment, OP-1206 was administered daily to diabetic rats in 3- and 30-mg/kg doses for 4 wk from the day of STZ injection. In the long-term study, 10 μg/kg OP-1206 was also given daily for 8 wk from 7 mo after induction of diabetes. The compound improved decreased sciatic motor nerve conduction velocity in both short-term and long-term diabetic rats. The nerve Na^+-K^+-ATPase activity of diabetic rats, reduced by 40% compared with controls, was reversed to the level of controls in both experiments, whereas weight loss and hyperglycemia were unchanged, and neither nerve sorbitol accumulation nor *myo*-inositol depletion was corrected. In a morphometric analysis of myelinated nerve fibers (MNFs) in long-term diabetes, the mean diameter of the largest 10% of MNFs was significantly reduced in untreated diabetic compared with control rats, but OP-1206 completely reversed this reduction. The results suggest that OP-1206 ameliorates a decrease in nerve Na^+-K^+-ATPase activity without any effect on nerve *myo*-inositol level and that the compound may be not only a potent therapeutic agent for the treatment of diabetic neuropathy but also a useful research tool to investigate the mechanism of nerve Na^+-K^+-ATPase activity regulation. *Diabetes* 40:726–30, 1991

Based on microvascular implications in the pathogenesis of diabetic neuropathy (1–9), it is natural to consider that prostaglandin E_1 (PGE_1) might be a useful therapeutic compound for the treatment of diabetic neuropathy (10,11), because PGE_1 has potent vasotropic effects, including vasodilation (12), inhibiting the aggregation of platelets (13), and increasing the deformability of erythrocytes (14). This compound has been clinically used and confirmed to have significant therapeutic effects on peripheral obstructive vascular diseases (15,16). Because of convenience of administration and long-lasting action, we have introduced an oral PGE_1 analogue, TFC 612, and reported that this compound improves reduced motor nerve conduction velocity (MNCV) and nerve blood flow (NBF) without normalizing hyperglycemia and abnormal sorbitol or *myo*-inositol metabolism in rats with both short-term and long-term streptozocin-induced diabetes (STZ-D) (10,11).

In our previous studies, the effect of PGE_1 on nerve Na^+-K^+-ATPase was not examined. A reduction in enzyme activity has been thought to be one of the main causes of experimental diabetic neuropathy, although, independent of enzyme activity, MNCV deteriorated in diabetic mice (17) and was improved with methylcobalamin in rats with STZ-D (8). Several therapeutic compounds, e.g., aldose reductase inhibitors (ARIs; 19), *myo*-inositol (20), and gangliosides (21), have been reported to reduce the decreases in this enzyme activity and nerve conduction velocity in experimental diabetic animals. Although TFC 612 does not correct abnormal sciatic nerve sorbitol or *myo*-inositol levels, the latter closely linked to nerve Na^+-K^+-ATPase activity, the possibility that this compound is able to improve decreased Na^+-K^+-ATPase activity independent of nerve *myo*-inositol level could not be ruled out. Such an improvement in enzyme activity without correction of *myo*-inositol level has been reported in rats with alloxan-induced diabetes treated with gangliosides (21).

In this study, we used another oral PGE_1 analogue, OP-1206 · α-CD (17S,20-dimethyl-*trans*-D_2-PGE_1 · α-cyclodextrin) (22,23). This compound has some advantages over TFC

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612 in that it has already been clinically used for peripheral obstructive arterial diseases, whereas TFC 612 has not. In addition, the former has a more physiologically compatible structure, one consisting only of carbon, hydrogen, and oxygen atoms, whereas the latter contains thio residue at the 17 position. In addition, side effects such as hypotension, flushing, nausea, and diarrhea are less frequent when the compound is given orally rather than infused intravenously.

This study was undertaken to confirm the effect of OP-1206 on long-term and short-term diabetic neuropathy and to ascertain whether this compound might improve nerve Na^+-K^+-ATPase activity in rats with both short-term and long-term STZ-D.

RESEARCH DESIGN AND METHODS

Two experiments were designed as follows. Experiment 1 was undertaken to evaluate the protective effects of OP-1206 (Ono, Osaka, Japan) on MNCV, nerve sorbitol and *myo*-inositol content, and the nerve Na^+-K^+-ATPase activity in short-term diabetic rats. Experiment 2 was undertaken to ascertain the same effects as well as effects on the morphology of myelinated nerve fibers (MNFs) in long-term diabetic rats. Male Sprague-Dawley rats were made diabetic at 11 wk of age in experiment 1 and at 8 wk of age in experiment 2 by a single injection of 40 or 45 mg/kg STZ, respectively, dissolved in citrate buffer, pH 4.5, into the tail vein. Diabetes was confirmed by glucosuria (>0.56 mM) and hyperglycemia (>19.4 mM) 3 days after the injection.

In experiment 1, animals were divided into four groups: nondiabetic control ($n=7$), untreated diabetic ($n=7$), diabetic treated with 3 $\mu g \cdot kg^{-1} \cdot day^{-1}$ OP-1206 (OP-3; $n=6$), and diabetic treated with 30 $\mu g \cdot kg^{-1} \cdot day^{-1}$ OP-1206 (OP-30; $n=7$). OP-1206 dissolved in 0.9% saline (5 ml/kg) was given daily to the OP-3 and OP-30 groups and 0.9% saline only to the control and untreated diabetic groups via gastric tubing from the day of STZ injection for 4 wk. MNCV was measured at the end of the experiment. Thereafter, animals were killed by decapitation, and blood samples were obtained. Immediately, both sciatic nerves were dissected, desheathed, weighed, frozen in liquid N_2, and kept at −70°C until assayed for sorbitol, *myo*-inositol, and Na^+-K^+-ATPase activity.

In experiment 2, animals were divided into three groups: nondiabetic control ($n=9$), untreated diabetic ($n=4$), and diabetic treated with 10 $\mu g \cdot kg^{-1} \cdot day^{-1}$ OP-1206 ($n=4$). Seven months after induction of diabetes, treatment was initiated via gastric tubing as in experiment 1 for 8 wk. Body weight was measured weekly, and plasma glucose levels were measured biweekly. Sciatic MNCV was measured at 0, 4, and 8 wk of treatment. At the end of the experiment, animals were killed, and both sides of the sciatic nerves were dissected in the same manner as in experiment 1 for assays of sorbitol, *myo*-inositol, and Na^+-K^+-ATPase activity. The left distal one-third of the sciatic nerve was used for morphometric analysis.

According to our preliminary experiment to determine the optimal therapeutic dosage of OP-1206 for diabetic neuropathy in rats, the minimum dose effective for MNCV was 3 μg/kg, whereas 10 and 30 μg/kg OP-1206 showed maximum effect on MNCV. This was the rationale for the dosages of OP-1206 used in this study.

MNCV was recorded from the right sciatic posterior tibial nerve conduction system with a modification of the method described by Sharma and Thomas (24). Animals were anesthetized with 35 mg/kg pentobarbital sodium, and their skin temperature was kept at 37°C with a heating lamp and pad. A stimulation electrode was inserted percutaneously in the sciatic notch and just above the internal malleus and stimulated supramaximally. Muscle action potentials were picked up from the plantar muscle and recorded by MS-92 (Medelec-Sanei, Tokyo).

Nerve sorbitol content was determined by the enzyme method of Bergmeyer et al. (25). The *myo*-inositol content was determined by the high-performance thin-layer chromatography technique of Stepanek (26) as described in detail elsewhere (10,11).

Nerve Na^+-K^+-ATPase activity was measured by quantification of liberated inorganic phosphate by the method of Fiske-Subbarow (27). The measurement was performed when samples were incubated in a buffer containing ATP with or without ouabain (27,28). Frozen nerve tissue was cut into fine segments and homogenized with a Physcotron (Niti-On Medical and Physical Instrument, Chiba, Japan) in 1 ml buffer containing 200 mM sucrose, 20 mM Tris-HCl, and 2 mM disodium EDTA (pH 7.5) at 4°C. One hundred microliters of the homogenate was incubated at 37°C for 20 min in 1 ml of buffer containing 130 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 30 mM histidine (pH 7.2), and 3 mM ATP with or without 1 mM ouabain (Merck, Darmstadt, Germany) after preincubation at 37°C for 10 min. The reaction was stopped with 200 μl of 60% perchloric acid and by placing tubes in ice for 10 min. Incubated mixtures were centrifuged at 1500 × *g* for 10 min. Ouabain-sensitive inorganic phosphate was determined by absorbance at 800 nm by a spectrophotometer (UV-265FW, Shimadzu) after reacting 500 μl of the supernatant with 480 μl of 2.5% molybdenum ammonium and 300 μl of Fiske-Subbarow's reductant for 20 min.

At the end of experiment 2, the left side of the distal one-third of the sciatic nerve was removed immediately after decapitation for morphometric analysis. The samples were fixed in 2.5% glutaraldehyde in 0.025 M cacodylate buffer, pH 7.38, at 10°C for 24 h. Tissue blocks were additionally fixed in 1% osmium tetroxide in the buffer at room temperature and embedded in epoxy resin. Transverse semithin sections (1 μm) were stained with toluidine blue. Morphometric analyses were performed with a computer-assisted digitizer. The smallest diameter of each MNF was measured on the screen of the digitizer connected with an optical microscope at a magnification of ×1800. The number and density of MNFs per fascicle, the median diameter of MNFs, the ratio of large MNFs (>7 μm diam) in the MNFs, and the mean diameter of the largest 10% of the MNFs were determined from each nerve sample. The number of fibers and the diameters were determined in three rectangular areas, two subperineurial and one centrifascicular for each fascicle, and systematically selected. More than 30% of the total fascicular area for each nerve was used for analysis. Because the MNF size histogram shows a bimodal pattern with a big trough ~7 μm in diameter, the fiber diameter of 7 μm was considered to clearly separate large from small myelinated fibers (29). The mean of the mean measurements for each animal was obtained for each group.

TABLE 1
Body weight, plasma glucose level, nerve sorbitol and *myo*-inositol content, and sciatic motor nerve conduction velocity (MNCV) in rats after treatment with OP-1206 · α-CD

				Sciatic nerve		
	n	Body weight (g)	Plasma glucose (mM)	Sorbitol (nmol/g wet wt)	*myo*-Inositol (μmol/g wet wt)	Sciatic MNCV (m/s)
Control	7	386 ± 10*	9.6 ± 1.3*	133 ± 39*	4.4 ± 1.5*	52.7 ± 3.2
Untreated diabetic	7	292 ± 25	29.9 ± 5.3	1278 ± 483	2.7 ± 0.6	45.9 ± 1.9†
OP-3	6	321 ± 38	31.6 ± 3.0	1158 ± 341	2.2 ± 0.4	49.1 ± 2.1
OP-30	7	277 ± 24	30.7 ± 3.9	1061 ± 233	2.7 ± 0.6	49.6 ± 1.9‡

Values are means ± SD. Diabetes was induced with streptozocin. OP-3 and OP-30, diabetic rats treated with OP-1206 · α-CD (3 or 30 μg · kg^{-1} · day^{-1}, respectively) for 4 wk. $P < 0.01$ for data for each parameter by 1-way analysis of variance.
*$P < 0.05$ vs. all diabetic groups.
†$P < 0.01$ vs. control.
‡$P < 0.05$ vs. untreated diabetic by Tukey's range test.

Most data were analyzed by one-way analysis of variance, followed by Tukey's range test. The few observations and variability of the method of measurement of Na^+-K^+-ATPase activity resulted in a failure to demonstrate a significant effect of OP-1206 in experiments 1 and 2 when this multiple analysis was used. Therefore, to answer the specific question of whether there was a significant effect of OP-1206 on diabetic nerves, comparisons were restricted to those between treated and untreated diabetic animals by unpaired Student's *t* tests. And statistical significance of any difference in MNCV in experiment 2 was evaluated by a two-way analysis of variance, followed by Tukey's range test. The difference was taken to be significant at $P < 0.05$.

RESULTS

In both experiments 1 and 2, none of the diabetic rats showed physiological body weight gain during the experiments (Tables 1 and 2). However, in contrast to a 20–30% reduction in body weight for the short-term diabetic rats, an ~50% reduction persisted throughout the treatment period for the long-term diabetic rats. Plasma glucose levels of diabetic rats showed an approximately threefold increase over those of control rats throughout both experiments. OP-1206 did not result in any particularly adverse reaction in either experiment.

In experiment 1, sciatic MNCV of untreated diabetic rats decreased by 7 m/s, a significant decrease compared with controls. Diabetic rats treated with 3 μg · kg^{-1} · day^{-1} OP-1206 showed improvement in MNCV, but the increase did not reach statistical significance ($0.05 < P < 0.1$). In contrast, 30 μg · kg^{-1} · day^{-1} OP-1206 significantly improved decreased MNCV in diabetic rats ($P < 0.05$).

At the start of the treatment in experiment 2, the sciatic MNCV of diabetic rats decreased by 10 m/s, a significant decrease compared with MNCV of control rats ($P < 0.05$; Table 3). MNCV in diabetic rats partially but significantly improved after 4 and 8 wk of treatment with OP-1206 but remained significantly less throughout the treatment period in untreated diabetic rats.

The sorbitol content of the sciatic nerve significantly increased and *myo*-inositol content significantly decreased for all diabetic groups in both experiments (Tables 1 and 2). OP-1206 treatment resulted in no changes in either experiment.

In experiment 1, nerve Na^+-K^+-ATPase activity was ~30% less in untreated diabetic rats than in controls (Table 4). The enzyme activity in the diabetic nerve after treatment with 30 μg · kg^{-1} · day^{-1} OP-1206 (the dose at which sciatic MNCV significantly improved) was significantly higher than for untreated diabetic rats ($P < 0.05$).

In experiment 2, the enzyme activity in untreated diabetic rats showed a significant reduction by 40% from that of controls, and the reduced enzyme activity was significantly reversed with OP-1206 treatment ($P < 0.05$ vs. untreated diabetic rats; Table 4).

The number per fascicle, the density, and the mean diameter of MNFs were not significantly different among groups (Table 5). The percentage of the large fibers (>7 μm diam) was 9% less for untreated diabetic than for control rats and became normal with OP-1206 treatment, although

TABLE 2
Body weight, plasma glucose, and nerve sorbitol and *myo*-inositol content in rats after treatment with OP-1206 · α-CD

				Sciatic nerve	
	n	Body weight (g)	Plasma glucose (mM)	Sorbitol (nmol/g wet wt)	*myo*-Inositol (μmol/g wet wt)
Control	9	540 ± 55	10.3 ± 0.9	357 ± 114	4.7 ± 1.0
Untreated diabetic	4	276 ± 38*	28.3 ± 1.2*	1615 ± 163*	3.3 ± 0.7†
OP-10	4	283 ± 35*	28.0 ± 1.8*	1778 ± 249*	3.4 ± 0.5†

Values are means ± SD. Diabetes was induced with streptozocin. OP-10, diabetic group treated with 10 μg · kg^{-1} · day^{-1} OP-1206 · α-CD. Data in all parameters show $P < 0.01$ by 1-way analysis of variance.
*$P < 0.01$, †$P < 0.05$, vs. control by Tukey's range test.

TABLE 3
Sciatic motor nerve conduction velocity (MNCV) in rats during OP-1206 · α-CD (OP) treatment

		Sciatic MNCV (m/s)		
	n	0	4 wk	8 wk
Control	9	60.3 ± 2.2	60.9 ± 1.5	60.1 ± 1.5
Untreated diabetic	4	50.7 ± 1.7*	49.1 ± 2.2*	49.0 ± 1.6*
OP-treated diabetic	4	50.5 ± 1.2*	55.2 ± 1.3*†	57.3 ± 1.9‡§

Values are means ± SD. Diabetes was induced with streptozocin.
*$P < 0.01$, ‡$P < 0.05$, vs. control.
†$P < 0.05$ vs. untreated diabetic by Tukey's range test after 2-way analysis of variance.
§$P < 0.01$ vs. untreated diabetic.

TABLE 4
Effect of OP-1206 · α-CD on nerve Na^+-K^+-ATPase activity in rats with short-term and long-term streptozocin-induced diabetes (STZ-D)

	n	Nerve Na^+-K^+-ATPase activity (μmol phosphate/mg protein)
Short-term diabetes		
Control	7	3.26 ± 0.70
Untreated diabetic	7	2.22 ± 0.74
OP-3	6	2.92 ± 0.60
OP-30	7	3.08 ± 0.57*
Long-term diabetes		
Control	8	3.02 ± 0.87
Untreated diabetic	4	1.82 ± 0.64
OP treated	4	2.97 ± 0.64*

Values are means ± SD. For short-term diabetes, 3 (OP-3) and 30 (OP-30) μg · kg^{-1} · day^{-1} OP-1206 · α-CD was administered to STZ-D rats for 4 wk from the day of induction of diabetes. For long-term diabetes, 10 μg · kg^{-1} · day^{-1} OP-12016 · α-CD was administered for 8 wk after 7 mo after injection of STZ.
*$P < 0.05$ vs. untreated diabetic by nonpaired Student's *t* test.

there was no statistically significant difference between any groups. On the other hand, the mean diameter of the largest 10% of the MNFs was significantly smaller in untreated than control rats ($P < 0.05$). OP-1206 completely reversed the decrease ($P < 0.05$ untreated vs. OP-1206–treated diabetic group).

DISCUSSION

In this study, an oral PGE_1 analogue, OP-1206, was shown to significantly improve decreased sciatic MNCV in rats with long-term and short-term STZ-D. This result is important because it shows that OP-1206 was effective even for chronic diabetic neuropathy with severe hyperglycemia of 7 mo duration and that the effect on MNCV continued to increase during 8 wk of treatment. We already reported such a long-lasting effect of PGE_1 analogue TFC 612 in rats with STZ-D of 3 mo duration (11).

The compound also completely prevented reduction in sciatic nerve Na^+-K^+-ATPase activity in short-term diabetes and reversed it in long-term diabetes. Enzyme activity is thought to play a critical role in nerve conduction and to be linked to nerve sorbitol and *myo*-inositol metabolism because ARI and *myo*-inositol supplementation have been reported to normalize impaired Na^+-K^+-ATPase activity and abnormal sorbitol and/or *myo*-inositol levels in rat sciatic nerve (19,20). However, the ameliorative effect of OP-1206 on enzyme activity is thought to be independent of nerve sorbitol and *myo*-inositol levels, because this compound does not change these levels in sciatic nerves and because the same results were obtained with another PGE_1 analogue, TFC 612 (10,11). The discrepancy between *myo*-inositol content and Na^+-K^+-ATPase activity has already been reported in galactose neuropathy where increased enzyme activity and decreased *myo*-inositol content of the sciatic nerve were noted (30) and in diabetic neuropathy treated with aldose reductase inhibitor, which prevents the accumulation of sorbitol and depletion of *myo*-inositol without ameliorating the Na^+-K^+-ATPase activity of the sciatic nerve in rats with STZ-D (31). Regarding the relationship between nerve *myo*-inositol content and Na^+-K^+-ATPase activity, Winegrad et al. (32) argued that the free–*myo*-inositol content in a nerve was not important but that a special labile pool of *myo*-inositol would be critical in nerve Na^+-K^+-ATPase regulation. However, because only the free form of *myo*-inositol is measurable, the importance of the *myo*-inositol pool remains to be elucidated.

What action mechanism of OP-1206 could explain the improvement in Na^+-K^+-ATPase activity of the sciatic nerve? First, ischemia is thought to lower the nerve phosphoinositide levels by the persistent activation of endogenous phospholipase C, thereby reducing membrane Na^+-K^+-ATPase (33). OP-1206 is able to increase NBF (unpublished observations) similar to TFC 612 (11), which improves endoneurial is-

TABLE 5
Morphometric data from myelinated sciatic nerve fibers in rats with long-term streptozocin-induced diabetes

		Myelinated nerve fibers				
	n	*n* (10^3/fascicle)	Density (10^3/mm^2)	Mean diameter (μm)*	Large fiber (%)†	Mean diameter of largest 10% (μm)‡
Control	8	4.18 ± 0.66	12.9 ± 1.66	6.2 ± 0.8	35.0 ± 15.4	9.47 ± 0.83
Untreated diabetic	4	4.48 ± 0.77	14.6 ± 1.96	5.7 ± 0.4	26.4 ± 10.1	7.95 ± 0.66§
OP10	4	5.20 ± 0.62	12.1 ± 0.76	6.0 ± 0.5	33.2 ± 7.9	9.53 ± 0.71‖

Values are means ± SD. OP-10, diabetic group treated with 10 μg · kg^{-1} · day^{-1} OP-1206 · α-CD.
*Mean of the mean diameter of myelinated nerve fibers determined as the shortest diameter for each group.
†Percentage of myelinated nerve fibers >7 μm diam.
‡Mean of the mean diameter of largest 10% of myelinated nerve fibers for each group.
§$P < 0.05$ vs. control.
‖$P < 0.05$ vs. untreated diabetic rats by Tukey's range test after 1-way analysis of variance.

chemia, resulting in amelioration of this enzyme activity. Second, PGE_1 is well known to promote cAMP production in platelets (34) and other tissues (35). cAMP may enhance inositol phospholipid metabolism especially through activating phosphatidylinositol kinase. Such a relationship between the cAMP and the inositol phospholipid systems has been reported in *Saccharomyces cerevisiae* (36). Enhanced phosphatidylinositide metabolism might thus be associated with the activation of protein kinase C, followed by the enhancement of Na^+-K^+-ATPase activity.

Finally, this compound might ameliorate the possible conformational abnormality of the enzyme resulting in normalization of the rate of dephosphorylation of ATP. Our preliminary study of nerve [^{3}H]ouabain binding, which reflects the content of Na^+-K^+-ATPase, seems to support the last hypothesis; in the same experimental design as experiment 1, this compound normalized the reduced enzymatic activity of the nerve in diabetic rats without affecting the number of the [^{3}H]ouabain binding sites (control 206.7 ± 69.5, untreated diabetic 223.0 ± 53.1, OP-3 210.1 ± 33.5, and OP-30 191.6 ± 45.0 pmol/g wet wt, respectively, measured by the method of Kjeldsen [37]).

In conclusion, OP-1206 had a significant effect on nerve Na^+-K^+-ATPase activity and MNCV in both short-term and long-term diabetic rats. In addition, the compound improved atrophy of MNFs in long-term diabetes. Because this compound has already been used clinically for peripheral vascular disease, our results support its clinical use for human diabetic neuropathy. Although the precise action mechanism of OP-1206 on diabetic neuropathy is still obscure, OP-1206 may be useful not only for the treatment of but also for research on the pathogenesis of diabetic neuropathy.

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