A Protein Kinase C-β–Selective Inhibitor Ameliorates Neural Dysfunction in Streptozotocin-Induced Diabetic Rats

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Increased protein kinase C (PKC) activity has been implicated in the pathogenesis of diabetic retinopathy and nephropathy. However, the role of PKC in diabetic neuropathy remains unclear. The present study was conducted to compare the effect of PKC inhibition by a PKC-β–selective inhibitor, LY333531 (LY), on diabetic nerve dysfunction with that of an aldose reductase inhibitor, NZ-314 (NZ). Streptozotocin-induced diabetic rats were treated with or without LY and/or NZ for 4 weeks, and motor nerve conduction velocity (MNCV), coefficient of variation of R-R interval (CVR-R), sciatic nerve blood flow (SNBF), peak latencies of oscillatory potentials on electroretinogram, PKC activities in membranous and cytosolic fractions of sciatic nerves, and polyol contents in the tail nerves were measured. Untreated diabetic rats demonstrated delayed MNCV, decreased CVR-R, reduced SNBF, and prolonged peak latencies of oscillatory potentials. Treatment with LY as well as NZ prevented all these deficits in diabetic rats. There were no significant differences in PKC activities in membranous or cytosolic fractions of sciatic nerves between normal and diabetic rats. Treatment with neither LY nor NZ altered PKC activities. Nerve *myo*-inositol depletion in diabetic rats was ameliorated not only by NZ, but also by LY. These observations suggest that inhibition of PKC-β by LY may have a beneficial effect in preventing the development of diabetic nerve dysfunction, and that this effect may be mediated through its action on the endoneurial micro-vasculature. *Diabetes* 48:2090–2095, 1999

Various hypotheses have been proposed to explain the pathogenesis of diabetic neuropathy, including increased polyol pathway activity, enhanced nonenzymatic glycation, protein kinase C (PKC) activation, and redox potential alterations (1). Among these theories, the role of polyol pathway hyperactivity in the development of diabetic neuropathy has been most extensively investigated, and the importance of this pathway has been confirmed by the beneficial effects of aldose reductase inhibitors (2,3). Increased polyol pathway– and hyperglycemia-induced *myo*-inositol depletion in the nerves causes an alteration of phosphoinositide turnover (4,5), which leads to a reduction in the availability of diacylglycerol (6,7), resulting in a decrease in the neuronal PKC activity. This diminished activity of PKC reduces the phosphorylation of Na^+-K^+-ATPase, which induces a deficit in nerve conduction and further nerve degeneration.

According to recent reports (8–12), on the other hand, diabetes and hyperglycemia induce an increase in the de novo synthesis of diacylglycerol, resulting in the activation of PKC in the retina, kidney, heart, and blood vessels (8). This increased PKC activity causes impaired retinal blood flow and renal hyperfiltration, contributing to the development of diabetic retinopathy and nephropathy (8). Among the many isoforms of PKC, the PKC-βII isoform has been reported to be predominantly activated by hyperglycemia in the retina, kidney, aorta, and heart of diabetic rats and in cultured rat mesangial cells (9–12). These observations have been confirmed by the fact that the PKC-β–selective inhibitor LY333531 (LY), a macrocyclic bisindolylmalemide compound, prevented these deficits in the retina and kidney (11,12).

Ischemia is considered to be one of the major factors contributing to the development of diabetic neuropathy (13,14). The reduction in endoneurial blood flow has been shown to be ameliorated by treatment with various vasodilatory agents, and this was accompanied by beneficial effects on neural functions in diabetic animals (14–19). The activation of vascular PKC is considered to lead to an impairment in vasodilation and an increase in vasoconstriction (20,21), which can occur in the endoneurial microvessels and cause a decrease in endoneurial blood flow, resulting in neural dysfunction in diabetes. Therefore, the inhibition of PKC may have a preventive effect on the development of diabetic neuropathy.

To investigate the effect of PKC inhibition on diabetic nerve dysfunction, physiological parameters such as motor nerve conduction velocity (MNCV) and nerve blood flow were measured in normal and streptozotocin-induced diabetic rats treated with or without the PKC-β–selective inhibitor LY (11). The effect of combined treatment with an aldose reductase inhibitor, NZ-314 (NZ) (22), was also examined.

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CVR-R, coefficient of variation of R-R interval; LY, LY333531; MNCV, motor nerve conduction velocity; NZ, NZ-314; PKC, protein kinase C; SNBF, sciatic nerve blood flow.

RESEARCH DESIGN AND METHODS

Animals. The 6-week-old male Wistar rats (Chubu Kagakushizai, Nagoya, Japan) with an initial body weight of 230–250 g were allowed to adapt to the experimental animal facility for 7 days. They were housed in an aseptic animal room at a temperature of 20–24°C and a humidity of 40–70%, with a 12-h lighting cycle and 12 fresh air changes per hour, and were allowed free access to rat diet and water. Diabetes was induced by a single injection of streptozotocin (50 mg/kg) (Sigma, St. Louis, MO), freshly dissolved in 50 mmol/l citric acid buffer (pH 4.5), into the tail vein of rats that had been fasted overnight. Control rats received an equal volume of citric acid buffer. At 1 week after streptozotocin administration, rats with plasma glucose concentrations of >16 mmol/l were selected as the diabetic rats. Normal rats were divided at random into three groups: untreated, NZ-treated, and LY-treated, and diabetic rats were divided into four groups: untreated, NZ-treated, LY-treated, and NZ + LY–treated. NZ-treated normal and diabetic rats received laboratory diet containing 0.05% NZ (~100 $mg \cdot kg^{-1} \cdot day^{-1}$) (Nippon Zoki Pharmaceutical, Osaka, Japan) for 4 weeks. LY (Eli Lilly, Indianapolis, IN) was administered to normal and diabetic rats at a dose of 50 mg/kg (suspended in 10% tragacanth gum solution) by oral gavage every day for 4 weeks. Untreated and LY-treated normal and diabetic rats had free access to laboratory diet and water. After the 4-week treatment, the following parameters were measured.

Measurement of caudal MNCV. Rats were placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C. After intraperitoneal injection of sodium pentobarbital (30–40 mg/kg), caudal MNCV was determined with a Neuropak NEM-3102 instrument (Nihon-Koden, Osaka, Japan) by the method of Miyoshi and Goto (23), as described previously (17).

Measurement of coefficient of variation of R-R interval. The coefficient of variation of R-R interval (CVR-R) was measured with a Labo-System ZS-501 (Fukuda ME, Tokyo) under mild anesthesia with 25 ml of ether to avoid the influence of other anesthetics, as described in our previous study (24).

Measurement of sciatic nerve blood flow. Under anesthesia with sodium pentobarbital, sciatic nerve blood flow (SNBF) was measured by the hydrogen clearance technique with an analog recorder BW-4 (Biochemical Science, Kanazawa, Japan) and electrolysis tissue blood flow meter RBA-2 (Biochemical Science), as described previously (17), and was calculated with the equation of Koshu et al. (25).

Measurement of electroretinogram. The rats were adapted to darkness for at least 20 min and then anesthetized by intraperitoneal injection of a mixture of 50 mg/ml ketamine (Ketalar 50; Sankyo Pharmaceutical, Tokyo), 25 mg/ml xylazine (Seructal; Bayer Japan, Tokyo), and physiological saline (10:1:11) at a dose of 0.2 ml/100 g body wt. Electroretinography was performed by the method of Segawa et al. (26), as described in our other studies (17,27). The peak latency was measured as the interval between stimulus onset and the peak of the corresponding b-wave, and the latencies were designated as O_1, O_2, O_3, and O_4 in order of superimposition on the b-wave, as described previously (17,26,27).

Measurement of PKC activity in the sciatic nerves. Freshly isolated and frozen sciatic nerves were powdered in liquid nitrogen and homogenized with a Dounce homogenizer (Wheaton Science Products, Millville, NJ). Cytosolic and membranous fractions were prepared by ultracentrifugation, and PKC activity was measured by its ability to transfer ^{32}P from [γ-^{32}P]ATP into the PKC-specific substrate, as described previously (9).

Measurement of polyol content in the tail nerves. Sorbitol, fructose, and *myo*-inositol contents in the tail nerves were measured by a trimethylsilylether-based gas-liquid chromatographic method, as described previously (28).

Statistical analysis. Results are presented as means ± SE. Differences among experimental groups were detected by analysis of variance, and the significance of differences between groups was assessed by Scheffe's *S* test. Significance was defined as a P value <0.05.

RESULTS

Body weight and biochemical data. Untreated diabetic rats demonstrated no body weight gain and remarkable hyperglycemia and hyperlipidemia compared with untreated normal rats. Treatment with either LY or NZ did not alter any of these parameters in normal and diabetic rats. The serum insulin concentration was significantly reduced in untreated diabetic rats compared with that in untreated normal rats, and was not affected by either LY or NZ in normal and diabetic rats (data not shown).

Nerve functions and SNBF. Figure 1 shows the effects of LY and/or NZ on MNCV, CVR-R, and SNBF. Untreated diabetic rats showed a significantly delayed MNCV compared with untreated normal rats (untreated diabetic: 22.1 ± 0.4 m/s, untreated normal: 34.2 ± 0.5 m/s, $P < 0.05$). Treatment with LY or NZ almost normalized the delay of MNCV in diabetic rats (LY: 31.8 ± 0.9 m/s, NZ: 30.1 ± 0.5 m/s). A significant decrease in CVR-R was observed in untreated diabetic rats compared with that in untreated normal rats (untreated diabetic: 1.08 ± 0.07%, untreated normal: 2.45 ± 0.26%, $P < 0.05$). The preventive effects of treatment with LY or NZ on the decreased CVR-R in diabetic rats were partial but significant (LY: 1.85 ± 0.12%, NZ: 2.18 ± 0.16%), and there were no significant differences in CVR-R between LY- or NZ-treated diabetic rats and untreated normal rats. LY and NZ had no effects on either MNCV or CVR-R in normal rats. The effects of the combination treatment with LY and NZ on MNCV (30.1 ± 0.55 m/s) or CVR-R (1.99 ± 0.16%) were similar to those of LY or NZ alone.

In untreated diabetic rats, SNBF was remarkably reduced compared with that in untreated normal rats (untreated diabetic: 2.6 ± 0.4 $ml \cdot min^{-1} \cdot 100\ g^{-1}$, untreated normal: 9.7 ± 0.4 $ml \cdot min^{-1} \cdot 100\ g^{-1}$, $P < 0.05$), and this reduction in SNBF was completely prevented by treatment with LY (10.4 ± 0.6 $ml \cdot min^{-1} \cdot 100\ g^{-1}$) or NZ (9.8 ± 0.6 $ml \cdot min^{-1} \cdot 100\ g^{-1}$). There were no significant differences in the effects on SNBF between LY or NZ alone and the combination with LY and NZ (9.1 ± 0.5 $ml \cdot min^{-1} \cdot 100\ g^{-1}$). Treatment with neither LY nor NZ affected SNBF in normal rats.

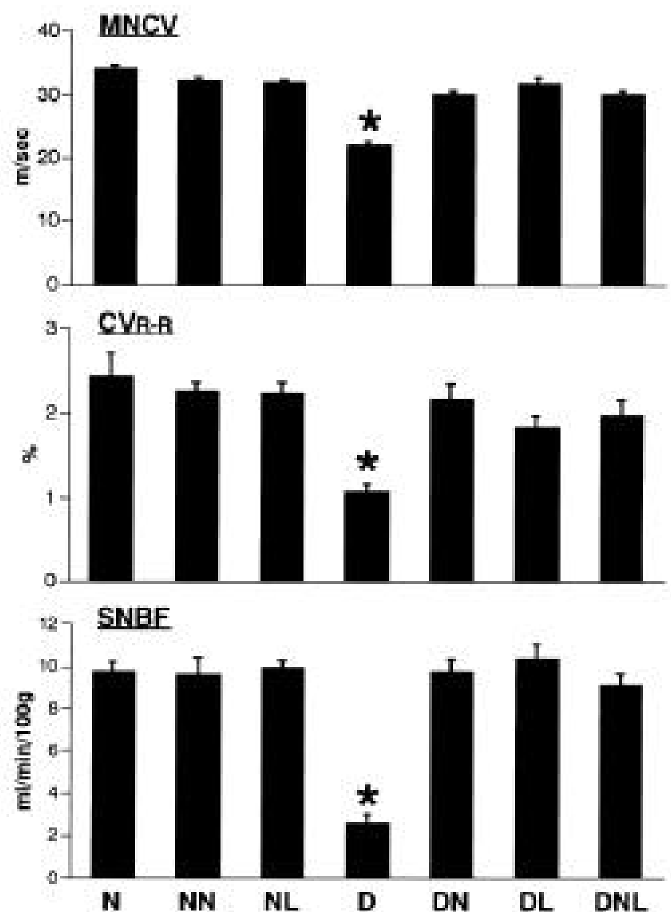

FIG. 1. MNCV, CVR-R on electrocardiogram, and SNBF in normal and diabetic rats treated with or without the aldose reductase inhibitor NZ and/or the PKC-β–specific inhibitor LY. D, untreated diabetic rats; DL, LY-treated diabetic rats; DN, NZ-treated diabetic rats; DNL, NZ + LY–treated diabetic rats; N, untreated normal rats; NL, LY-treated normal rats; NN, NZ-treated normal rats. Data are means ± SE. *P < 0.05 vs. all other groups.

TABLE 1
The peak latencies of the oscillatory potentials on electroretinogram in normal and diabetic rats treated with or without the aldose reductase inhibitor NZ and/or the PKC-β–specific inhibitor LY

	n	O_1 (ms)	O_2 (ms)	O_3 (ms)	O_4 (ms)	(O_1–O_4) (ms)
Normal rats						
Untreated	11	23.9 ± 0.6	35.2 ± 0.3	45.8 ± 0.7	58.0 ± 0.8	162.8 ± 1.1
NZ-treated	10	23.1 ± 0.9	35.6 ± 0.7	45.2 ± 0.6	57.2 ± 0.7	161.0 ± 1.4
LY-treated	12	22.4 ± 0.4	33.1 ± 0.7	44.0 ± 1.1	55.3 ± 0.8	154.8 ± 2.4
Diabetic rats						
Untreated	11	25.9 ± 1.1	39.7 ± 0.7*	55.5 ± 0.7*	70.9 ± 0.7*	191.9 ± 1.9*
NZ-treated	11	22.3 ± 0.7	36.9 ± 0.9	47.8 ± 1.5	62.5 ± 1.3	171.7 ± 2.0
LY-treated	11	20.9 ± 0.4	34.0 ± 0.7	46.3 ± 1.3	58.7 ± 1.4	159.9 ± 2.7
NZ + LY–treated	12	20.8 ± 0.6	34.8 ± 0.7	48.2 ± 0.7	63.4 ± 0.9	167.1 ± 1.7

Data are means ± SE. *$P < 0.05$ vs. all other groups.

Peak latencies of oscillatory potentials on electroretinogram. Although there were no significant differences in the peak latency of the oscillatory potential, O_1, between each experimental group, untreated diabetic rats demonstrated a significant prolongation of the peak latencies of individual oscillatory potentials O_2, O_3, and O_4, as well as that of the summed potential ($O_1 + O_2 + O_3 + O_4$). Prolongation of these latencies was significantly reduced by treatment with LY and/or NZ. No effects of LY or NZ on the peak latencies were observed in normal rats (Table 1).

PKC activity in the sciatic nerves. Although the mean values of PKC activities in the membranous fraction of diabetic nerves were less than those of normal nerves, there were no statistically significant differences in the specific activities of PKC either in the membranous or in the cytosolic fraction between untreated normal and diabetic rats. Treatment with neither NZ nor LY significantly altered the PKC activities in normal or diabetic rats (Table 2). Although the PKC activities in NZ + LY–treated diabetic rats were not measured in this study, it can be speculated that there would be no effect.

Polyol contents in the tail nerves. Untreated diabetic rats demonstrated a remarkable accumulation of sorbitol and fructose and a significant depletion of the *myo*-inositol content compared with untreated normal rats. Treatment with NZ partially but significantly reduced the sorbitol and fructose accumulation and almost normalized the *myo*-inositol content in diabetic rats. On the other hand, treatment with LY increased the *myo*-inositol content without affecting the sorbitol or fructose accumulation in diabetic rats. The combined treatment of LY with NZ in diabetic rats did not alter the effect of NZ on the sorbitol, fructose, or *myo*-inositol contents (Table 3). Treatment with neither LY nor NZ affected the polyol contents in normal rats.

DISCUSSION

The present study demonstrated that treatment of diabetic rats with a PKC-β–selective inhibitor, LY, or an aldose reductase inhibitor, NZ, ameliorated deficits in nerve functions such as delayed MNCV and decreased CVR-R, which were accompanied by an increase in endoneurial blood flow. These effects of NZ are consistent with those found in previous studies conducted with other aldose reductase inhibitors (29–32). According to the hypothesis in which the hyperglycemia-induced polyol pathway hyperactivity represents one of the pathogenic mechanisms of diabetic neuropathy (4), it can be predicted that PKC activity is decreased in the nerve tissues and that the inhibition of PKC-β activity by LY might cause a further deterioration of nerve functions, which is in conflict with the results of the present study. However, it remains controversial whether the PKC activity in diabetic nerve tissues is decreased, unchanged, or increased (33–37), and it also remains unclear which isoforms of PKC in diabetic nerve tissues are altered. Therefore, it can also be the case that the PKC-β inhibition by LY may have no effects on nerve functions.

Kim et al. (33) demonstrated that the composite PKC activity in the cytosolic fraction of diabetic nerves was decreased and that this decrease was *myo*-inositol sensitive. They also observed that a PKC agonist or *myo*-inositol supplementation normalized the decreased Na^+-K^+-ATPase activity in sciatic nerves of diabetic rats (38). In addition, recently, Kowluru et al. (35) reported that the PKC activity in sciatic nerves is decreased not only in diabetic, but also in galactosemic rats. In contrast, there are no reports in which increased PKC activity has been shown by direct measurement of the specific activity in diabetic nerve tissues. Previous reports demonstrated that treatment with PKC inhibitors such as calphostin C and H7 restored the decrease in Na^+-K^+-ATPase activity in sciatic nerves of diabetic mice (39,40), which would support the PKC activation theory for the pathogenesis of diabetic neuropathy. However, MNCV was not measured in these reports.

TABLE 2
PKC activity in the membranous and cytosolic fractions of sciatic nerves of normal and diabetic rats treated with or without the aldose reductase inhibitor NZ and/or the PKC-β–specific inhibitor LY

	n	Membranous ($pmol \cdot min^{-1} \cdot mg^{-1}$)	Cytosolic ($pmol \cdot min^{-1} \cdot mg^{-1}$)
Normal rats			
Untreated	5	213.8 ± 51.9	21.4 ± 5.6
NZ-treated	5	218.2 ± 28.9	20.6 ± 4.7
LY-treated	5	210.9 ± 29.7	10.3 ± 1.2
Diabetic rats			
Untreated	5	189.1 ± 32.8	14.0 ± 3.1
NZ-treated	5	185.4 ± 39.4	20.8 ± 4.4
LY-treated	5	193.0 ± 12.2	18.5 ± 1.9

Data are means ± SE.

TABLE 3
Sorbitol, fructose, and *myo*-inositol contents in tail nerves of normal and diabetic rats treated with or without the aldose reductase inhibitor NZ and/or the PKC-β–specific inhibitor LY

	n	Sorbitol (nmol/mg)	Fructose (nmol/mg)	*myo*-Inositol (nmol/mg)
Normal rats				
Untreated	11	0.40 ± 0.05	1.48 ± 0.14	4.89 ± 0.27
NZ-treated	10	0.40 ± 0.04	1.26 ± 0.11	4.93 ± 0.26
LY-treated	12	0.33 ± 0.02	1.13 ± 0.07	4.73 ± 0.29
Diabetic rats				
Untreated	11	3.33 ± 0.27*	6.35 ± 0.78*	2.82 ± 0.26*
NZ-treated	11	2.41 ± 0.12†	4.67 ± 0.28†	3.99 ± 0.25
LY-treated	11	2.98 ± 0.29*	6.08 ± 0.25*	4.05 ± 0.17
NZ + LY–treated	12	2.27 ± 0.30†	4.59 ± 0.42†	4.33 ± 0.74

Data are means ± SE. *$P < 0.05$ vs. normal rats; †$P < 0.05$ vs. normal and untreated diabetic rats.

Although the galactosemic animals show a delayed MNCV that is similar to that found in diabetic rats, the Na^+-K^+-ATPase activity in the nerve tissues of this animal model is not decreased (41–44). These observations suggest that the restoration of Na^+-K^+-ATPase activity is not by itself sufficient to cause an amelioration in the nerve dysfunction of diabetes. Thus, it is not clear whether the restoration of Na^+-K^+-ATPase activity by PKC inhibition leads to the prevention or improvement of diabetic nerve dysfunction. Therefore, this is the first report that indicates a beneficial effect of PKC inhibition on the development of diabetic nerve dysfunction by demonstrating an amelioration of the abnormalities in nerve functions, including the delayed MNCV and decreased CVR-R.

In contrast to diabetic neuropathy, PKC-β activation through an increase in de novo synthesis of diacylglycerol has recently been considered to contribute to the development of diabetic retinopathy and nephropathy (8), which is supported by the following observations. PKC-β activity is increased in vascular tissues such as retina, heart, aorta, and renal glomeruli of diabetic animals and in cells cultured with elevated glucose concentrations such as endothelial cells, smooth muscle cells, and mesangial cells (8). Treatment of streptozotocin-induced diabetic rats with the PKC-β inhibitor LY prevented the decrease in retinal blood flow and the increases in glomerular filtration rate and urinary albumin excretion rate (11,12). Prolongation of the peak latencies of the oscillatory potentials on electroretinogram is a specific indicator of retinal dysfunction in the early stage of diabetic retinopathy (45,46). The finding obtained in the present study that treatment of diabetic rats with LY prevented this deficit in electroretinogram would confirm the importance of PKC-β activation in the development of diabetic retinopathy.

It has been reported that treatment with various kinds of vasodilatory agents prevents the deficit in nerve functions by increasing the endoneurial blood flow without restoring the metabolic alterations in diabetic nerves (14–19), and it has been suggested that endoneurial ischemia may be a major contributing factor in the development of diabetic neuropathy. Based on the previous studies described above, it can be easily speculated that vascular tissues in the endoneurium as well as the retina and kidney of diabetic rats would possess increased PKC-β activity, resulting in vasoconstriction and a decrease in endoneurial blood flow. As PKC-β inhibition exerted beneficial effects on the abnormalities in retinal and renal hemodynamics (11,12), inhibition of PKC-β activity by LY would also increase the endoneurial blood flow, leading to an amelioration of nerve functions. The results obtained in this study would support this hypothesis.

PKC activity in neither the membranous nor the cytosolic fractions of the sciatic nerves was significantly altered in diabetic rats compared with that in normal rats in this study. However, from this result, it cannot be concluded that the neural components, such as Schwann cells and axons, of diabetic nerves have PKC activity similar to that in normal nerves. Considering that the PKC activity in the vascular component of diabetic nerves would be higher than that in normal nerves, this observation still suggests that the PKC activity in neural components may be decreased in diabetic rats. In addition, the PKC-specific substrate used in the present study, which is derived from epidermal growth factor receptor, has relatively high specificities to PKC-α, -β, and -γ and a low specificity to PKC-ε (47). Tissue-specific changes in PKC isoforms have been reported; PKC-βI and -βII are activated in diabetic glomeluri (12) and aortas (9), respectively. PKC isoforms other than PKC-α, -β, and -γ, such as PKC-ε, may be altered in neural components of diabetic nerves, which might cause no differences in PKC activity between normal and diabetic nerves in this study. According to the report by Borghini et al. (36), the isoforms of PKC-α, -βII, and -δ are mainly present in the Schwann cell compartment, and PKC-ε and -βI in the axonal compartment. Therefore, PKC-β inhibition by LY should decrease PKC activity in vivo. However, no significant decreases in PKC activity were observed in either normal or diabetic rats treated with LY. In this study, the PKC activity was measured in vitro after many procedures, which might cleave the binding of LY to the enzyme, resulting in no inhibition of PKC activity by LY. It remains unclear whether LY did ameliorate diabetic nerve dysfunction as the PKC-β inhibitor or something else without a direct demonstration of the PKC inhibition by LY in nerve tissues. However, it can be highly expected by the previous report (10) and our unpublished observations (J.N., Y.K., Y.H., K.K., Y.Y., H.K., N.H.), in which the glucose-induced increase in PKC activity in cultured endothelial cells and/or smooth muscle cells is inhibited by LY, that the inhibitory effect of LY on PKC activity would be exerted in endoneurial microvasculature.

One of the interesting findings in this study is that PKC-β inhibition in diabetic rats prevented *myo*-inositol depletion without altering sorbitol or fructose accumulation. Although

the precise mechanisms for this effect of LY are unknown, it may be mediated through an increase in the energy-dependent *myo*-inositol uptake into nerve cells, which would be due to the increased energy production by an amelioration of nerve blood flow. A similar effect was observed in our previous study conducted with niceritrol, which does not inhibit polyol pathway but has a vasodilatory action (48).

The effect of combined treatment with an aldose reductase inhibitor and a PKC inhibitor has never been investigated. The beneficial effect of polyol pathway inhibition by NZ was not abolished by PKC-β inhibition by LY, suggesting that the polyol pathway–induced inhibition of PKC activity would not involve PKC-β or that the decreased activity of isoforms of PKC other than PKC-β may be a major contributing factor in the development of diabetic nerve dysfunction.

In conclusion, it still remains unclear whether the PKC activity in the neural component of diabetic nerve tissues is decreased, unchanged, or increased. However, it was clearly demonstrated that the inhibition of PKC-β activity by LY prevents the development of diabetic nerve dysfunction in rats. Although further investigation of the precise mechanisms of this effect by LY will be required, it is suggested that the preventive effect of LY on diabetic nerve dysfunction could be mediated through its action on the endoneurial microvasculature.

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