Gliclazide Inhibits Diabetic Neuropathy Irrespective of Blood Glucose Levels in Streptozotocin-Induced Diabetic Rats

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N-acetylcysteine and pentoxifylline, free radical scavengers and inhibitors of tumor necrosis factor-α (TNF-α) production, inhibit the development of peripheral neuropathy in streptozotocin (STZ)-induced diabetic rats. This study was designed to elucidate the effect of gliclazide, an oral hypoglycemic sulfonylurea, on diabetic neuropathy, because it has been indicated to be a free radical scavenger and TNF-α inhibitor. Rats were fed with powder chow mixed with gliclazide or glibenclamide as a control ad libitum. Blood glucose levels and body weight were remarkably higher and lower in diabetic than in nondiabetic rats, respectively, while gliclazide and glibenclamide had no effect on these in both diabetic and nondiabetic rats throughout a 24-week experiment. Serum lipoperoxide levels and lipopolysaccharide (LPS)-induced serum TNF-α activities were significantly increased in diabetic rats, whereas these were significantly inhibited in gliclazide-treated rats. Motor nerve conduction velocity (MNCV) of the tibial nerve significantly slowed in diabetic rats compared with nondiabetic rats. On the other hand, the slowed MNCV was significantly inhibited in gliclazide-treated diabetic rats after 16 experimental weeks. Morphometric analysis showed that gliclazide prevented decreased myelinated fiber area ($P < .05$), increased fiber density ($P < .001$), and decreased axon/myelin ratio ($P < .05$) in diabetic rats. Glibenclamide treatment did not affect serum lipoperoxide, TNF-α, MNCV, or nerve morphology in this experiment. These results indicate that gliclazide has a beneficial effect on peripheral neuropathy in STZ-induced diabetic rats, irrespective of blood glucose levels.

DIABETIC NEUROPATHY may be caused by nerve and vascular dysfunctions due to elevated activity of the polyol pathway.[1] In addition, advanced glycation end products produced under chronic hyperglycemia, which stimulate cytokine and free radical productions, may play a role in the pathogenesis of diabetic neuropathy.[2] We have previously reported that the production of tumor necrosis factor-α (TNF-α) was significantly enhanced under long-term hyperglycemia in diabetic animal models[3,4] and humans,[5] and that *N*-acetylcysteine[6] and pentoxifylline,[7] both known as free radical scavengers and inhibitors of TNF-α production, ameliorated the diabetic neuropathy in streptozotocin (STZ)-induced diabetic rats. These results imply that free radical scavengers and/or TNF-α inhibitors may suppress the development of diabetic neuropathy.

Gliclazide, an oral hypoglycemic sulfonylurea, is widely used for the treatment of non–insulin-dependent diabetes mellitus.[8] In addition to stimulating insulin secretion, gliclazide has a variety of bioactivities, eg, scavenging free radicals,[9] inhibition of TNF-α production (Fukuzawa M, Satoh J, and Qiang X, et al, submitted), and inhibition of prostanoid release and platelet aggregation both in animals and humans.[10] This indicates that gliclazide may inhibit diabetic neuropathy, as does *N*-acetylcysteine[6] and pentoxifylline.[7] In this study, we investigated whether gliclazide has an inhibitory effect on diabetic neuropathy using STZ-induced diabetic rats. Glibenclamide was used as a control, because it is also a sulfonylurea, but not a free radical scavenger[9,10] or TNF-α inhibitor (Fukuzawa M, Satoh J, Qiang X, et al, submitted).

MATERIALS AND METHODS

Animals and Induction of Diabetes

Twelve-week-old male Wistar/Slc rats (purchased from Clea Japan, Tokyo, Japan) with a mean body weight of 270 g were used for the experiment. Rats were induced diabetes by intravenous injection of STZ (30 mg/kg body weight) from the tail vein and defined as diabetic when the blood glucose level was greater than 300 mg/dL (16.8 mmol/L) 24 hours after the STZ injection.

Administration of Gliclazide and Glibenclamide

Low-dose (L; 0.025%) and high-dose (H; 0.1%) gliclazide (produced by Les Laboratoires Servier, Neuilly, France and provided by Dainippon Pharmaceutical, Osaka, Japan) or 0.00625% glibenclamide (purchased from Seloc AG, Liestal, Switzerland) was mixed with powder chow and orally given ad libitum during the 24-week experiment. Doses of drug were determined based on a previous report[11] and common clinical doses for humans, eg, 40 to 160 mg/body weight (50 kg)/d (0.8 to 3.2 mg/kg/d) of gliclazide and 2.5 to 10 mg/body weight (50 kg)/d (0.05 to 0.2 mg/kg/d) of glibenclamide. Rats were administered gliclazide or glibenclamide at doses approximately 10 to 40 times as much as those for humans, ie, 28.2 to 100.7 mg/kg/d of gliclazide or 7.6 mg/kg/d of glibenclamide (Table 1). Rats were divided to five groups, each consisting of 10 rats: nondiabetic (nonDM), nonDM/gliclazide-H, diabetic (DM), DM/gliclazide-H, DM/gliclazide-L, and DM/glibenclamide. The actual dose of gliclazide or glibenclamide intake was calculated from the amount of chow eaten daily. The serum concentration of gliclazide and glibenclamide of each rat was measured at the end of the experiment by high-performance liquid chromatography in Teijin Biolaboratories (Tokyo, Japan).

Measurement of Blood Glucose, Body Weight, and Motor Nerve Conduction Velocity

Body weight, blood glucose, and motor nerve conduction velocity (MNCV) were measured at 0, 4, 12, 16, and 20 weeks of the 24-week experiment. Blood glucose levels were measured by a glucometer (Glutest-E; Kyoto Daiichi Kagaku, Kyoto, Japan) using a glucose oxidase method. MNCV was measured in the left tibial nerve by a standard method previously reported.[12]

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Table 1. Serum Concentration of Gliclazide or Glibenclamide, and Serum Insulin and Blood Glucose Levels

Rats	N	Drugs			Serum Insulin (ng/mL)	Blood Glucose (mg/dL)
		Content in Power Chow (%)	Real Dose Administered (mg/kg/d)	Serum Gliclazide or Glibenclamide (μg/mL)		
nonDM	6	0	0	0	2.38 ± 1.13	80.7 ± 9.3
nonDM/Gliclazide-H	9	0.1	50.2 ± 2.9	9.2 ± 1.3	2.14 ± 0.75	83.8 ± 7.0
DM	9	0	0	0	0.71 ± 0.16*	334.5 ± 33.6†
DM/gliclazide-L	5	0.025	28.2 ± 2.8	2.5 ± 1.3	0.64 ± 0.17*	342.3 ± 28.1†
DM/gliclazide-H	7	0.1	100.7 ± 11.9	10.8 ± 4.1	0.75 ± 0.22*	332.3 ± 53.6†
DM/glibenclamide	4	0.00625	7.6 ± 0.3	0.8 ± 0.4	0.69 ± 0.19*	367.5 ± 33.0†

NOTE. Sera were obtained at the end (24 weeks) of the experiment.
*$P < .01$, †$P < .001$ *v* nonDM.

Assay of Serum Lipoperoxide

Lipoperoxide was assayed in serum obtained at the end of experiment (24 weeks) by the thiobarbiturate reaction using a commercial kit (Lipoperoxide-Test Wako; Wako, Osaka, Japan). Briefly, malondialdehyde derived from serum lipoperoxide under the acidic condition was reacted with thiobarbiturate, and malondialdehyde-thiobarbiturate complex extracted with n-butanol was detected by absorption of 553 nm of fluorescence.[13]

Assay of Serum Insulin

Serum obtained at the end (24 weeks) of the experiment was assayed for rat insulin using a commercial kit (Rat insulin [^{125}I] assay system with magnetic separation; Amersham Life Science, Buckinghamshire, England).

Assay of TNF-α Activity

At 22 weeks of the 24-week experiment, each rat was injected with lipopolysaccharide (LPS; 0.4 mg/kg) from the tail vein; 90 minutes later, blood was sampled and the serum was isolated and measured for TNF-α activity using LM cells, a subline of the TNF-sensitive mouse fibroblast (L929), as previously reported.[14]

Morphological Analysis of Nerve

At the end (24 weeks) of the experiment, each rat was anesthetized with ether, and the left sciatic nerve was dissected and immediately fixed with 2.5% glutaraldehyde fixative. These specimens were embedded in Epon and semithin cross sections were cut and stained with toluidine blue for light microscopic examination and morphometric analysis. The stained nerve sections were photographed by a digital camera (Olympus BH-2; Olympus Optical, Tokyo, Japan) equipped with a television camera (Nikon DS-505; Nikon, Tokyo, Japan). The fascicular area, in which the perineurium was used as a border, myelinated fiber number, and myelinated fiber area were measured and analyzed by a computer-assisted image analysis system (NIH Image; Agfa Arcusscanner connecting with Power Macintosh 8500).

Statistical Analysis

All data are expressed as means ± SD. Statistical significance of differences was calculated by ANOVA and the Bonfferoni correction for independent samples. *P* values ≤.05 were considered significant.

RESULTS

Effect of Gliclazide Administration on Blood Glucose Levels and Body Weight

The blood glucose levels of diabetic rats were more than 300 mg/dL 24 hours after the STZ injection. The hyperglycemia remained stable throughout the entire experiment (Fig 1). There was no difference in blood glucose levels between nontreated rats and gliclazide- or glibenclamide-treated rats both in diabetic and nondiabetic rats, ie, administration of gliclazide or glibenclamide had no effect on blood glucose levels in this experiment.

Before the experiment, all rat groups had almost similar body weights. Body weight increased with aging in nonDM and nonDM/gliclazide-H rats (Fig 2). On the other hand, DM, DM/gliclazide-H, DM/gliclazide-L, and DM/glibenclamide rats lost body weight and their weight was significantly lighter than that of nonDM rats ($P < .001$). Administration of gliclazide or glibenclamide had no effect on body weight (Fig 2).

Inhibition With Gliclazide Administration of Increased Serum Lipoperoxide Levels in Diabetic Rats

Serum lipoperoxide levels were significantly ($P < .05$) increased in DM rats as compared with nonDM rats. The increased lipoperoxide levels were significantly ($P < .05$) inhibited in DM/gliclazide-H, but not in DM/glibenclamide rats (Fig 3).

Inhibition With Gliclazide Administration of Enhanced LPS-Induced Serum TNF-α Activity

As shown in Fig 4, LPS-induced serum TNF-α activities were markedly enhanced in DM rats as compared with those in

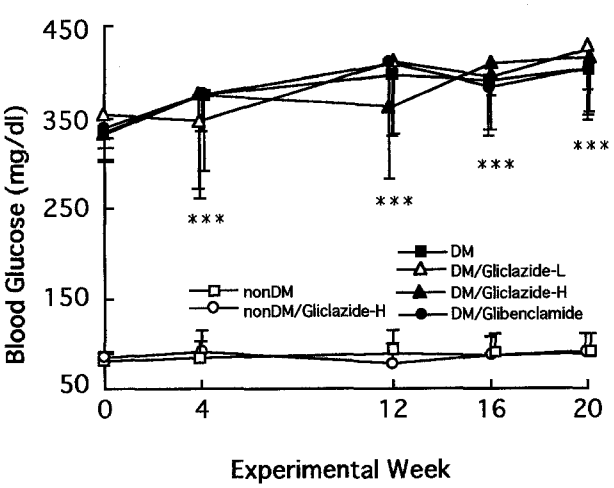

Fig 1. Effect of gliclazide on blood glucose levels. ***$P < .001$, nondiabetic rats *v* diabetic rats.

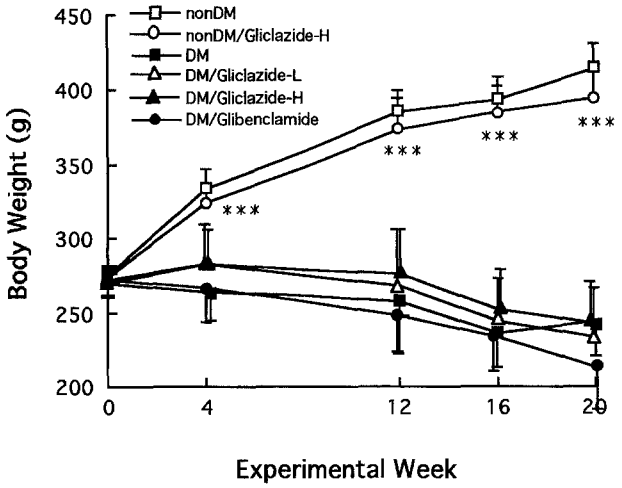

Fig 2. Effect of gliclazide on body weights. ***$P < .001$, nondiabetic rats *v* diabetic rats.

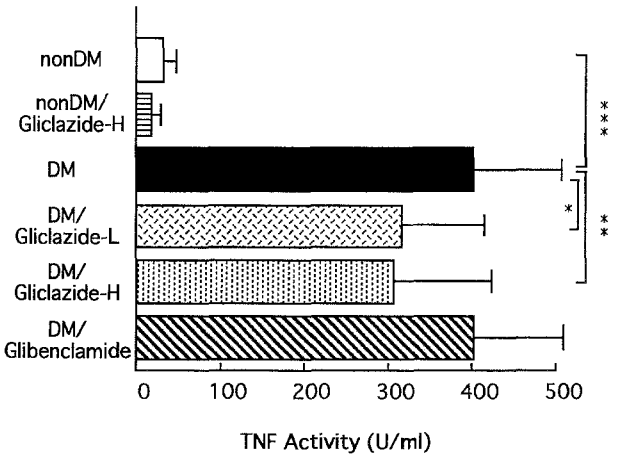

Fig 4. Inhibition with gliclazide administration of enhanced TNF-α production in diabetic rats. Sera were obtained at 22 weeks of the 24-week experiment. *$P < .05$, **$P < .01$, ***$P < .001$, *v* DM rats.

nonDM rats. On the other hand, the enhancement of TNF-α activity in DM rats was significantly inhibited in both DM/gliclazide-H ($P < .01$) and DM/gliclazide-L ($P < .05$), but not DM/glibenclamide rats. Gliclazide administration did not significantly inhibit TNF-α activity in nondiabetic rats (nonDM/gliclazide-H).

Effects of Gliclazide Administration on MNCV

MNCV slightly increased with aging in nonDM rats, whereas it significantly decreased in DM rats after 4 weeks of the experiment. However, the slowing of MNCV in diabetic rats was significantly ($P < .05$) inhibited after the 16th experimental week in DM/gliclazide-H and DM/gliclazide-L rats, but not in DM/glibenclamide rats (Fig 5). Gliclazide administration did not affect MNCV in nondiabetic rats (nonDM/gliclazide-H).

Effects of Gliclazide Administration on Nerve Morphology

At the end of the 24-week experiment, the left tibial nerve was morphologically analyzed. As shown in Fig 6, the mean myelinated fiber area and axon/myelin ratio were significantly diminished in DM rats as compared with those in nonDM rats, and these diminutions were significantly inhibited in DM/gliclazide-H rats, but not in DM/glibenclamide rats. Inversely, the mean fiber density was significantly increased in DM rats as compared with that in nonDM rats, but the increase was inhibited in DM/gliclazide-H rats. The mean fascicular area was significantly decreased in DM rats, as well as the other groups (DM/gliclazide-H and DM/glibenclamide), but not significantly compared with that in nonDM rats.

Doses Administered and Serum Levels of Gliclazide and Glibenclamide, and Serum Insulin and Blood Glucose Levels at the End of the Experiment

Table 1 shows the drug contents in powder chow, the actual doses administered and the serum levels of gliclazide and glibenclamide, and serum insulin and blood glucose levels in the nonfasting condition in the rats randomly chosen at the end of the 24-week experiment. The data indicate that serum levels of drugs were closely correlated with the content of the drugs in

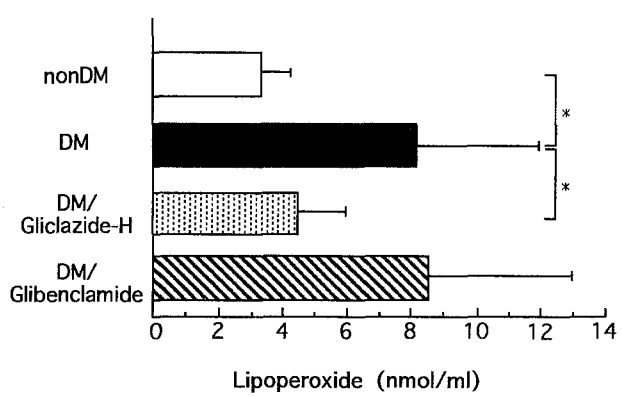

Fig 3. Inhibition with gliclazide administration of increased serum lipoperoxide levels in diabetic rats. Sera were obtained at the end (24 weeks) of the experiment. *$P < .05$, *v* DM rats.

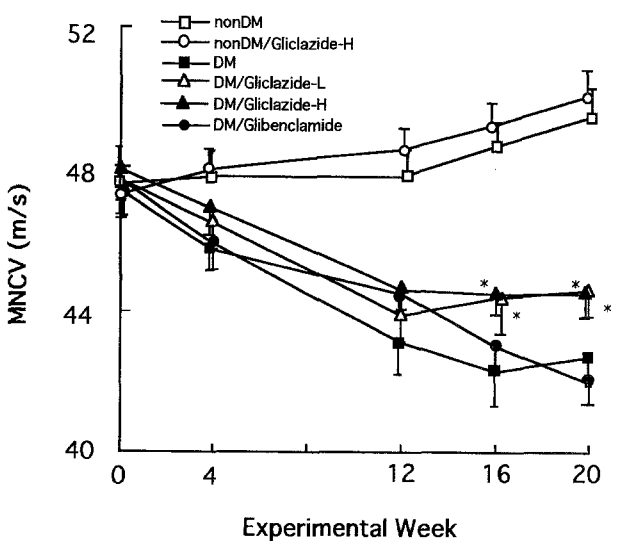

Fig 5. Effects gliclazide administration on MNCV. *$P < .05$, *v* DM rats. $P < .01$ (4 weeks) and $P < .001$ (12, 16, and 20 weeks) between nondiabetic rats and diabetic rats.

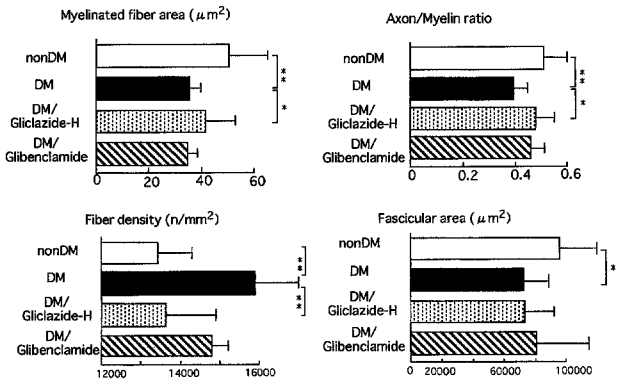

Fig 6. Effect of gliclazide administration on nerve fiber morphology. Nerve tissues were obtained at the end (24 weeks) of the experiment. *$P < .05$, **$P < .01$, ***$P < .001$, *v* DM rats. Difference not significant between DM and DM/gliclazide.

the powder chow in diabetic rats, the rats were maintained well with these drugs, and sufficient serum levels of the drugs[9-11] were maintained throughout the experiment. However, neither gliclazide nor glibenclamide increased serum insulin levels in diabetic rats, although nonfasting insulin levels were significantly lower in diabetic rats than those in nondiabetic rats (Table 1).

DISCUSSION

In the present study, we used same methods as for our previous reports on *N*-acetylcysteine[6] and pentoxifylline,[7] and found that the administration of gliclazide inhibited the increased serum lipoperoxide levels (Fig 3), the enhanced production of TNF-α (Fig 4), and the progression of diabetic neuropathy (Fig 5 and 6).

Rats were chronically administered gliclazide mixed with chow. Doses of gliclazide administered (28.2 to 100.7 mg/kg/d) (Table 1) were extremely higher than clinical doses for humans, but equivalent to the rat experiments previously reported,[8,11] while serum levels of gliclazide (2.5 to 10.8 μg/mL) (Table 1) were comparable to those in both rats[11] and humans,[8] which effectively lowered blood glucose levels in the short-time experiment. However, gliclazide or glibenclamide administration did not affect nonfasting serum insulin or glucose levels in either nondiabetic and diabetic rats (Table 1), although the exact reason is unclear. A possible reason is that the rats were chronically administered sulfonylureas with foods, which might induce secondary failure to sulfonylureas.[15] Similarly, gliclazide did not affect body weight, which increased in nondiabetic rats, while it decreased in diabetic rats (Fig 2). There was no difference in serum gliclazide levels between nonDM/gliclazide-H and DM/gliclazide-H rats, although the actual dose administered was larger in DM/gliclazide-H than nonDM/gliclazide-H rats (Table 1). The reason may be due to polyphagia and polyuria in diabetic rats.

The slowed MNCV in diabetic rats was significantly prevented by gliclazide treatment (Fig 5). Although gliclazide administration was begun at the start of the experiment, the improvement of MNCV by gliclazide was statistically significant after the 16th experimental week (Fig 5). This implies that gliclazide administration may affect the later phase of neuropathy, ie, inhibiting exacerbation of the neuropathy, rather than preventing initial events. The beneficial effect of gliclazide on diabetic neuropathy was also confirmed by morphological analyses at the end of experiment. Nerve fiber area, axon/myelin ratio, and fascicular area decreased, while nerve fiber density increased in diabetic rats as compared with nondiabetic rats (Fig 6), as previously reported.[12,16] These abnormalities were significantly suppressed in gliclazide-treated rats, except for the fascicular area, although the reason is unclear (Fig 6).

The exact mechanism(s) of action by which gliclazide inhibited neuropathy is unknown. Recent studies have demonstrated that gliclazide is a powerful free radical scavenger.[9] Free radical scavengers prevent peripheral neuropathy in diabetic rats.[17] The effect of gliclazide as a free radical scavenger is also shown in our results, ie, the increased serum lipoperoxide level in diabetic rats was inhibited by gliclazide treatment (Fig 3). In addition, the enhanced TNF-α production in diabetic rats was also significantly inhibited in rats administered with high and low doses of gliclazide (Fig 4). Scavenging of free radicals and TNF-α inhibition with gliclazide might have ameliorated diabetic neuropathy, possibly by suppressing impairment of microcirculation induced by free radicals and/or TNF-α.[18,19] However, the relative contribution of these effects to the amelioration of neuropathy is obscure, even if these effects are ascribed to the beneficial effect of gliclazide. It has been reported that free radical scavengers inhibited the decrease in MNCV even after 1 month,[17] whereas gliclazide had no effect on MNCV for the same period. This might be due to weak free radical scavenging activity of gliclazide. On the other hand, administration of glibenclamide had no effect on the increased serum lipoperoxide level (Fig 3), enhanced TNF-α production (Fig 4), slowed MNCV (Fig 5), or abnormal morphology of the nerve (Fig 6) in STZ-induced diabetic rats, although glibenclamide, as well as gliclazide, is one of the sulfonylureas and serum levels of glibenclamide were maintained sufficiently for biological activities (Table 1).[9,10] Therefore, it seems that gliclazide is a unique sulfonylurea with an activity that suppresses the development of diabetic neuropathy.

In conclusion, administration of gliclazide significantly inhibited the slowing of MNCV and morphological changes of the peripheral nerve in diabetic rats, irrespective of blood glucose levels in STZ-induced diabetic rats.

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