Decreased Blood Glucose Excursion by Nateglinide Ameliorated Neuropathic Changes in Goto-Kakizaki Rats, an Animal Model of Non-obese Type 2 Diabetes

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In the present study, we examined the effect of long-term suppression of postprandial hyperglycemia and glycemic fluctuation in Goto-Kakizaki (GK) rats, a type 2 diabetic animal model, by nateglinide (NG), a fast-acting hypoglycemic agent, on some measures of neuropathy and compared the outcome with the slow-acting effect of glibenclamide (GC). GK rats fed twice daily were given NG (50 mg/kg) or GC (1 mg/kg) orally before each meal for 24 weeks. The dose of NG and GC was determined by the data of their comparable suppressive effects on hyperglycemia as a total sum of glucose values after glucose load. At the end, there was no significant influence of treatment with NG or GC on body weight, fasting blood glucose, and glycated hemoglobin in GK rats. However, NG treatment suppressed postprandial hyperglycemia by 50% throughout the observation period, whereas this effect was not apparent in GC-treated rats. Delayed motor nerve conduction velocity was normalized by NG treatment, while GC had a partial (50%) effect. GK rats showed elevated contents of sorbitol and 3-deoxyglucosone in the sciatic nerve, and these changes were inhibited by NG treatment. Reduced Na^+/K^+-adenosine triphosphatase (ATPase) activity in GK rats was not affected by either NG or GC treatment. These results suggest that meticulous control of postprandial hyperglycemia is essential to inhibit the development of neuropathy in type 2 diabetes.

THE DIABETES CONTROL and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) have shown that rigorous glycemic control is important for the prevention of microvascular and macrovascular complications.[1,2] In addition to elevated fasting glucose levels, postprandial hyperglycemia is a characteristic feature of type 2 diabetes, and recent studies have shown that fluctuation of blood glucose is implicated in the pathogenesis of diabetic complications.[3,4]

Nateglinide (NG) is one of the meglitinide oral hypoglycemic agents, which induces rapid insulin secretion by pancreatic β cells and thus suppresses postprandial hyperglycemia in patients with type 2 diabetes.[5-8] NG is therefore expected to reduce the daily fluctuations of blood glucose in patients with type 2 diabetes, allowing better glycemic control with less risk of hypoglycemia than sulfonylurea compounds.[6-8] It is not known, however, whether reducing the fluctuation of blood glucose by lowering the postprandial glucose level with NG indeed prevents or inhibits the progression of diabetic complications in type 2 diabetes.

The Goto-Kakizaki (GK) rat is an animal model of spontaneous-onset, non-obese type 2 diabetes, featuring postprandial hyperglycemia, impaired insulin secretion, progressive reduction of β-cell mass, and the development of long-term diabetic complications.[9-12] Given a diet restricted twice daily, this model exhibits apparent postprandial hyperglycemia and fluctuation of the circadian blood glucose. In the present study, we investigated the long-term effect of reducing blood glucose fluctuation in GK rats by NG under diet restriction on some measures of neuropathy and compared the outcome with the effect of glibenclamide (GC).

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Submitted February 1, 2002; accepted April 22, 2002.

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0026-0495/02/5111-0045$35.00/0
doi:10.1053/meta.2002.35195

MATERIALS AND METHODS

Animals

This study was reviewed and approved by the Animal Care and Use Committee of Ajinomoto. Male GK rats and normal Wistar rats were obtained from Charles River Japan (Yokohama, Japan) at 6 weeks of age, and each rat was housed in a polycarbonate cage with a wooden chip mat on the floor. Water was available ad libitum, and standard rat chow (22.6% protein, 53.8% carbohydrate, 5.6% fat, 6.6% mineral and vitamin mixture, and 3.3% fiber, total: 356 kcal/100 g) (CRF-1, Charles River Japan, Yokohama, Japan) was provided under time restriction as described below. The animal room was kept on a 12-hour light/dark cycle (7 AM to 7 PM/dark, 7 PM to 7 AM/light), with a temperature range of 22°C ± 1°C and a relative humidity of 55% ± 5% throughout the experimental period.

Diet Restriction and Drug Administration

For precise evaluation of the influence of postprandial hyperglycemia on the development of neuropathic changes, rats were trained to consume the diet chow in 1 hour that was provided twice a day under the dark period (10 AM to 11 AM and 4 PM to 5 PM). The animals were acclimatized to laboratory conditions for about 2 weeks until blood glucose levels reached a plateau. After this dietary conditioning, the daily blood glucose profile showed a marked postprandial increase in the range of 3.0 to 8.0 mmol/L (mean ± SE, 6.03 ± 1.1 mmol/L) in GK rats, while there was no apparent change in Wistar rats (Fig 1). At 14 weeks of age, GK rats were divided into 3 groups (n = 9 each) and were given NG (50 mg/kg), GC (1 mg/kg), or vehicle alone (0.5% methylcellulose) by oral gavage twice daily just before each meal for 24 weeks. The doses of NG and GC were determined from previous data that showed a similar glucose-lowering effect after oral administration to fasting normal rats.[13] To confirm the comparable effects of NG and GC at this dose, GK rats were given 50 mg/kg NG or 1 mg/kg GC shortly before 1 g/kg oral glucose tolerance test (OGTT). Blood sampling at OGTT was performed at 0, 15, 30, 60, 90, 120, 180, and

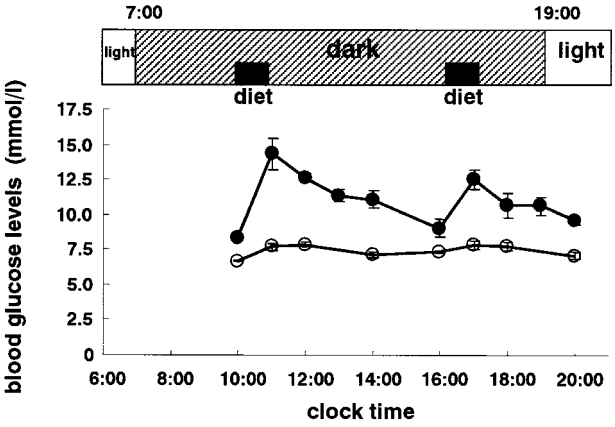

Fig 1. Daily changes of blood glucose after diet consumption in normal Wistar rats (n = 3) (○) and GK rats (n = 3) (●). Diet was provided as indicated at the top of the graph. Data are expressed as means ± SEM.

240 minutes for the measurement of blood glucose and immunoreactive insulin (IRI).

Blood Glucose, Glycated Hemoglobin, and Plasma Insulin Levels

Blood glucose and glycated hemoglobin were monitored throughout the experimental period. Blood sampling from the tail vein was performed before the first meal of the day (at 10 AM after 17 hours fasting) to determine the fasting blood glucose, as well as 1 hour after the first meal of the day (at 11 AM) to measure the postprandial blood glucose. Blood glucose and glycated hemoglobin values were examined by the glucose oxidase method using an autoanalyzer (Fuji Dri-Chem 5500, Tokyo, Japan) and by a commercial kit (Glicaffin; Seikagaku-Kogyo, Tokyo, Japan), respectively. Plasma insulin was measured using an insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga, Takamatsu, Japan).

Motor Nerve Conduction Velocity

Motor nerve conduction velocity (MNCV) was measured in the caudal nerve as described previously.[14] During measurement, the tail temperature was maintained at 38°C ± 1°C in a heated liquid paraffin bath controlled by a thermostat under diethylether anesthesia.

Nerve Biochemistry

At the end of treatment, the sciatic nerve was excised from each rat after 17 hours fasting (18 hours after the last drug administration), and Na^+/K^+-adenosine triphosphatase (ATPase) activity, concentrations of sorbitol, and 3-deoxyglucosone (3-DG) were determined. For measurement of Na^+/K^+-ATPase activity, the sciatic nerve was homogenized in 20 mmol/L Tris-HCl, pH 7.5 containing 200 mmol/L sucrose. Na^+/K^+-ATPase activity was determined based on the method described previously.[15] Ouabain-sensitive fraction was represented as an activity of Na^+/K^+-ATPase. For measurement of sorbitol and 3-DG, the sciatic nerve was homogenized in phosphate-buffered saline, pH 7.4 and deproteinized by adding perchloric acid. Sorbitol concentrations were determined by spectrofluorometry with sorbitol dehydrogenase.[15] 3-DG concentrations were measured by ELISA (Uchida Y, et al, submitted for publication).

Statistical Analysis

Statistical analysis was performed with StatView for Windows version 5.0 (SAS Institute, Cary, NC). All data are expressed as means ± SEM. Student's *t* test was used for comparison between untreated GK rats and normal Wistar rats to confirm occurrence of diabetes-induced changes. To evaluate the effects of each drug, comparisons among groups of GK rats were performed by 1-way analysis of variance (ANOVA), followed by Dunnett's post hoc test, in which untreated GK rats were used as the control group. Correlation coefficients were calculated by linear regression analysis. A *P* value of less than .05 was considered to be significant.

RESULTS

Hypoglycemic Effects of NG and GC on GK Rats

On OGTT, there was a marked increase of blood glucose in GK rats, which was sustained at 10 mmol/L levels 240 minutes after glucose challenge (Fig 2) . This sharp increase 30 and 60 minutes after glucose load was significantly suppressed by NG, but not GC. By contrast, lowering effects of blood glucose became conspicuous in GC-treated GK rats 180 and 240 minutes after glucose load. Consequently, summation of glucose-lowering effects (glucose $AUC_{0\text{-}240}$) was almost equivalent

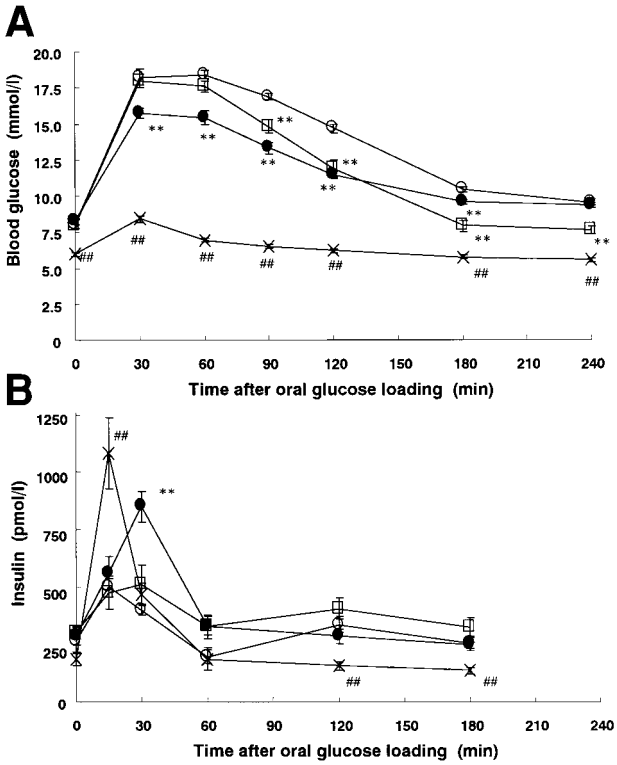

Fig 2. Effects of NG and GC on blood glucose levels after oral glucose administration in GK rats. After 17 hours fasting, 50 mg/kg NG, 1 mg/kg GC, or vehicle alone was administered just before glucose (1 g/kg) load, and changes of (A) blood glucose and (B) plasma insulin were monitored. GK, vehicle-treated GK rats (n = 9) (○); GK+GC, glibenclamide-treated GK rats (n = 9) (□); GK+NG, nateglinide-treated GK rats (n = 9) (●); Wistar, normal Wistar rats (n = 9) (×). Data are expressed as means ± SEM, n = 9 for each group. ##*P* < .01 *v* GK (by Student's *t* test); *******P* < .01 *v* GK (by Dunnet's test).

Table 1. Glucose and Insulin AUC After Oral Glucose Loading (1 g/kg) With Each Drug

	Glucose $AUC_{0\text{-}240\ min}$ (mmol · h/L)	Insulin $AUC_{0\text{-}60\ min}$ (pmol · h/L)	Insulin $AUC_{0\text{-}180\ min}$ (pmol · h/L)
GK	55.0 ± 0.7	358.7 ± 24.9	922.0 ± 71.4
GK+GC	47.6 ± 1.4*	429.5 ± 48.1	1,164.3 ± 124.0
GK+NG	47.0 ± 0.8*	578.2 ± 48.5*	1,155.0 ± 87.0
Wistar	25.6 ± 0.2†	517.2 ± 62.0‡	837.3 ± 101.7

NOTE. $AUC_{a-b\ min}$, total sum of the values from time a to b. Data are expressed as means ± SEM.

Abbreviations: GK, vehicle-treated GK rats (n = 9); GK + GC, glibenclamide-treated GK rats (n = 9); GK+NG, nateglinide-treated GK rats (n = 9); Wistar, normal Wistar rats (n = 9).

*$P < .01$ *v* GK (by Dunnet's test).

†$P < .01$ *v* GK (by Student's *t* test).

‡$P < .05$ *v* GK.

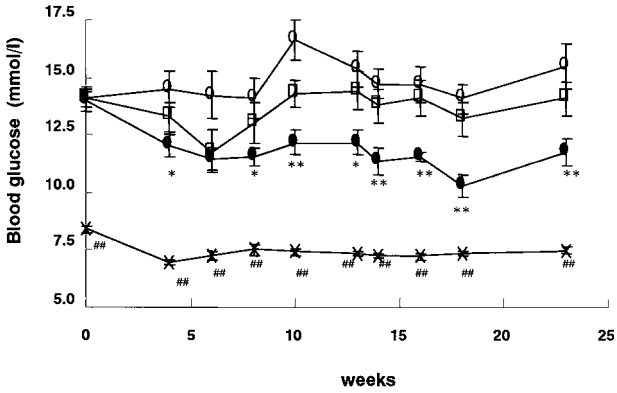

Fig 3. Changes of postprandial blood glucose in vehicle-treated GK rats (n = 9) (○), GC-treated GK rats (n = 9) (□), NG-treated GK rats (n = 9) (●), and normal Wistar rats (n = 9) (×) during the experimental period. Postprandial blood glucose was measured at 1 hour after the first meal (11 AM) of each day. Data are expressed as means ± SEM. ##$P < .01$ *v* GK (by Student's *t* test); *$P < .05$ *v* GK; **$P < .01$ *v* GK (by Dunnet's test).

between NG and GC-treated groups (Table 1). However, glycemic fluctuation was more severe in the GC-treated group than the NG-treated group (Fig 2). In accordance with blood glucose changes, there was an early-phase insulin secretion with a peak at 30 minutes in NG-treated GK rats, whereas induced insulin secretion was rather attenuated and prolonged in GC-treated GK rats (Fig 2). Nevertheless, total insulin secretion (insulin $AUC_{0\text{-}180}$) induced by these 2 compounds was similar (Table 1).

Laboratory Data

Laboratory data of GK rats treated with NG and GC for 24 weeks are summarized in Table 2. Average food consumption was approximately 15 to 19 g/d for GK rats and 18 to 20 g/d for Wistar rats, which corresponded to approximately 80% of the amounts during ad libitum feeding. Mean body weights of untreated and treated GK rats were lower at week 24 compared with a group of normal Wistar rats ($P < .01$). There was no significant difference in body weight at the end among the groups of treated and untreated GK rats.

Postprandial blood glucose values of untreated GK rats were about 2-fold higher than those of Wistar rats throughout the experimental period (Fig 3). There was marked suppression of the postprandial hyperglycemia in NG-treated GK rats ($p < .01$) (Fig 3), while there was no influence on fasting blood glucose in these animals (Table 2). In contrast, the effect of lowering the postprandial hyperglycemia was not significant in the GC-treated group, although average values were lower than those in untreated GK rats (Fig 3). There were no significant differences of glycated hemoglobin values among the 3 groups of GK rats (Table 2). Basal plasma insulin concentrations were higher in GK rats than in normal Wistar rats and were not influenced by drug treatment (Table 2).

MNCV and Nerve Biochemistry

Untreated GK rats showed a 15% decrease in MNCV when compared with Wistar rats ($P < .01$). NG treatment restored the MNCV to normal ($P < .01$ *v* untreated GK rats), while GC

Table 2. Laboratory Data of Experimental Animals

	Body Weight (g)	Food Intake (g/d)	Fasting Blood Glucose (mmol/L)	Glycated Hemoglobin (%)	Basal Insulin (pmol/L)
GK					
Initial	285.1 ± 4.7	15.9 ± 1.4	9.76 ± 0.39	5.41 ± 0.15	263.6 ± 45.7
End	383.2 ± 7.6	18.7 ± 2.2	9.17 ± 0.12	5.58 ± 0.28	264.3 ± 52.6
GK+GC					
Initial	282.1 ± 7.8	16.9 ± 0.9	10.16 ± 0.82	5.14 ± 0.09	179.6 ± 50.1
End	388.9 ± 6.3	17.0 ± 1.6	8.54 ± 0.38	5.47 ± 0.20	303.0 ± 27.9
GK+NG					
Initial	290.0 ± 7.0	15.4 ± 1.8	9.87 ± 0.50	5.40 ± 0.18	220.8 ± 53.3
End	384.8 ± 10.2	18.4 ± 0.8	9.38 ± 0.18	5.25 ± 0.13	287.7 ± 34.8
Wistar					
Initial	380.6 ± 6.5*	18.2 ± 0.8	5.49 ± 0.09*	3.69 ± 0.15*	80.5 ± 24.0*
End	493.3 ± 7.6*	19.9 ± 1.2	6.41 ± 0.12*	3.81 ± 0.10*	188.6 ± 30.3

NOTE. Data are expressed as means ± SEM.

Abbreviations: GK, vehicle-treated GK rats (n = 9); GK + GC, glibenclamide-treated GK rats (n = 9); GK + NG, nateglinide-treated GK rats (n = 9); Wistar, normal Wistar rats (n = 9).

*$P < .01$ *v* GK (by Student's *t* test).

treatment showed only partial (≈ 50%) improvement ($P < .05$ *v* untreated GK rats) (Fig 4A).

The sorbitol content of the sciatic nerve was elevated 2-fold in untreated GK rats, while treatment with NG inhibited this accumulation ($P < .05$ *v* untreated GK rats) (Fig 4B). Inhibition of sorbitol accumulation in GC-treated rats did not reach significance. There was a 1.6-fold increase of the sciatic nerve 3-DG content in untreated GK rats when compared with Wistar rats ($P < .005$) (Fig 4C). NG treatment reduced the accumulation of 3-DG by 50% ($P < .05$ *v* untreated GK rats), whereas GC treatment did not show a significant decrease.

Na^+/K^+-ATPase activity of the sciatic nerve was decreased by 80% in untreated GK rats ($P < .05$ *v* normal Wistar rats). Restoration of this activity was not apparent in either NG- or GC-treated rats (Fig 4D).

When postprandial blood glucose values, nerve sorbitol, nerve 3-DG, and MNCV at the end of experiment were examined for their interrelationships, postprandial blood glucose values correlated well with the accumulation of sorbitol in the sciatic nerve ($r = .65$, $P < .0001$, Fig 5A). Nerve sorbitol contents were closely correlated with nerve 3-DG contents ($r = .89$, $P < .0001$, Fig 5B). Postprandial blood glucose values were also correlated with reductions of MNCV ($r = -.54$, $P = .0009$, Fig 5C), whereas there was no significant correlation between fasting blood glucose values and MNCV (data not shown).

DISCUSSION

The present study demonstrated that GK rats fed twice a day showed a marked fluctuation of the circadian blood glucose, accompanied by increased contents of nerve sorbitol and 3-DG, and reductions of MNCV and Na^+/K^+-ATPase activity. Suppression of postprandial hyperglycemia and glycemic excursion was found to be more apparent in the NG-treated group than in the GC-treated group. The better glycemic control in NG-treated GK rats was reflected by the greater improvement of MNCV and biochemical changes in the peripheral nerve. In the present study, we compared the effects of suppression of postprandial hyperglycemia by NG with those by GC. The doses of both drugs (50 mg/kg NG and those of 1 mg/kg GC) were determined based on our previous data that showed the comparable hypoglycemic effects in normal rats[13] and on the current results from OGTT. Despite a slight tendency to lower fasting blood glucose levels, 1 mg/kg GC treatment did not have any significant influences on postprandial glucose levels. There still remains a possibility, however, that a larger dose of GC might have affected blood glucose levels and might have been more efficacious on neuropathic changes. Nevertheless, current results strongly implicate that postprandial glucose excursion plays an important role in the development of the neuropathic changes in type 2 diabetes and could be the therapeutic target to prevent diabetic complications. The dietary protocol adopted in this study enabled us to examine the role of repeated fluctuations of blood glucose in the pathogenesis of diabetic complications.

GK rats are known to exhibit progressive neuropathic changes characterized by reduced MNCV, decreased ouabain sensitive Na^+/K^+-ATPase activity, and teased nerve fiber abnormalities.[11] Recent experiments have shown that both vascular and metabolic factors are involved in the pathogenesis of

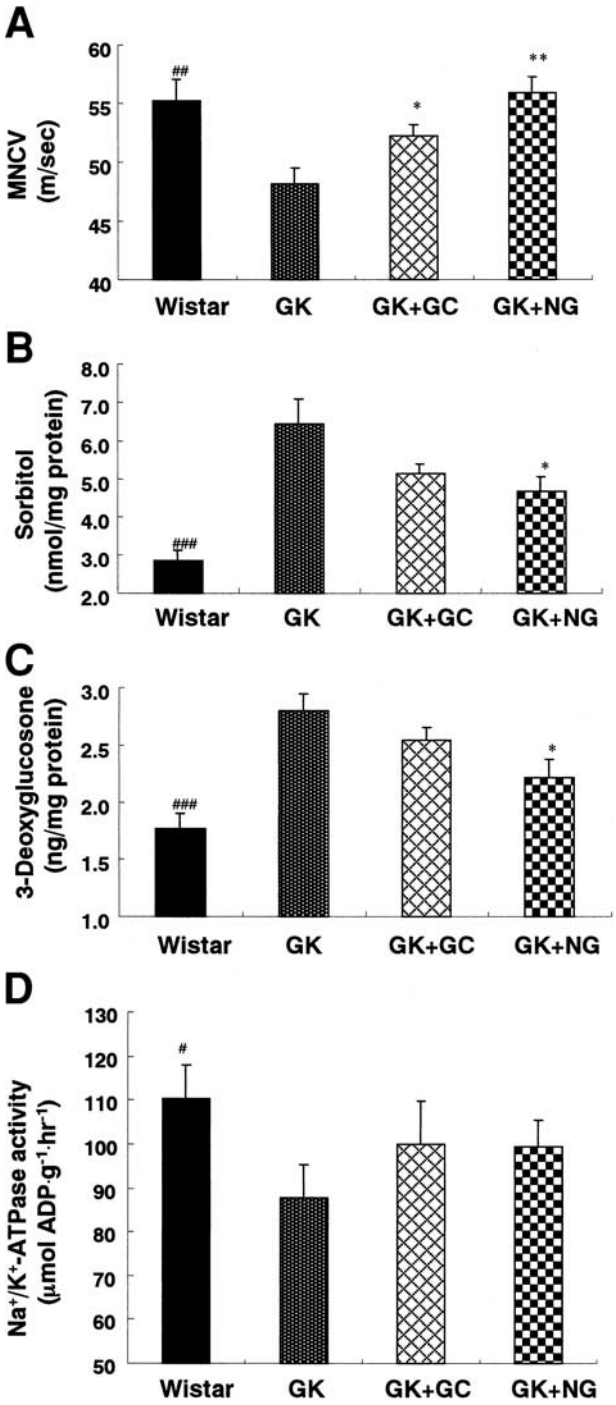

Fig 4. (A) MNCV, (B) nerve sorbitol, (C) nerve 3-DG content, and (D) ouabain-sensitive Na^+/K^+-ATPase activity in the sciatic nerve at the end of treatment (week 24). GK, vehicle-treated GK rats (n = 9); GK+GC, glibenclamide-treated GK rats (n = 9); GK+NG, Nateglinide-treated GK rats (n = 9); Wistar, normal Wistar rats (n = 9). Data are expressed as means ± SEM. $^{\#}P < .05$ *v* GK; $^{\#\#}P < .01$ *v* GK; $^{\#\#\#}P < .005$ *v* GK (by Student's *t* test); $^{*}P < .05$ *v* GK; $^{**}P < .01$ *v* GK (by Dunnet's test).

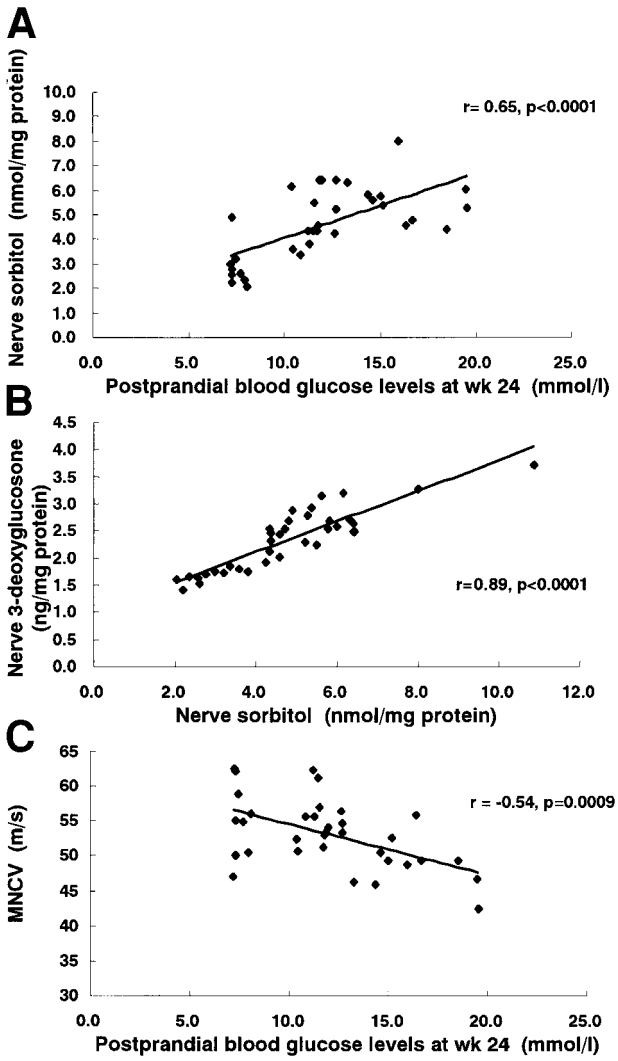

Fig 5. Correlations between (A) postprandial blood glucose and nerve sorbitol contents (r = .65, P < .0001), (B) nerve sorbitol and 3-DG contents (r = 0.89, P < .0001), and (C) postprandial blood glucose and MNCV (r = -.54, P < .0001) at the end of treatment (week 24).

diabetic neuropathy. Early reduction of MNCV is closely associated with neurovascular dysfunction triggered by enhanced polyol pathway,[16] excessive production of glycated proteins,[17] increased protein kinase C activity,[18] and excessive oxidative stress.[15] The improvement of neuropathic changes in GK rats treated with NG may be related to the smaller excursions of postprandial blood glucose, resulting in reduced flux of the polyol pathway, less accumulation of glycation products, and inhibition of oxidative stress. This hypothesis is supported by the significant reduction of nerve sorbitol and 3-DG contents in NG-treated GK rats. In contrast, the effects of GC were not sufficient to inhibit the accumulation of sorbitol and 3-DG with only partial recovery of MNCV.

Inhibition of neuropathic changes by NG treatment may also be related to improvement of insulin and C-peptide secretion. It has been shown that the neuropathic patterns are different between animal models of type 1 and type 2 diabetes.[19,20] In type 1 models, nerve axonal atrophy and expanded vascular spaces are the predominant structural changes,[21,22] whereas overall fiber atrophy and narrowed vascular lumina are observed in the peripheral nerves of type 2 models.[23] The difference in the pattern of neuropathy between these 2 types has been ascribed to the action of insulin and C-peptide on the peripheral nerves.[24] C-peptide has been shown to ameliorate the decrease of MNCV and nerve Na^+/K^+-ATPase activity in insulin-deficient diabetic rats.[24,25] It is likely, however, that rather than the total amount of insulin release, but physiologic secretion in response to glucose intake has contributed to the improvement of neuropathic changes, because the total amounts of insulin released by NG and GC were similar between NG- and GC-treated groups.

There is a close relationship between increased polyol pathway, excessive glycation, and oxidative stress.[26] The increase of nerve sorbitol and 3-DG detected in this study confirmed the activation of the polyol pathway and subsequent enhancement of nonenzymatic glycation in the peripheral nerves in GK rats. Recently, increased glucose oxidation with superoxide production in the mitochondria was proposed to have a central role in the pathogenesis of diabetic complications.[27] Our study demonstrated a close relationship between sorbitol and 3-DG contents and also showed that postprandial blood glucose levels were correlated with both accumulation of sorbitol in the sciatic nerve and reduction of MNCV, reinforcing the importance of metabolic factors and subsequent neurovascular dysfunction affected by blood glucose fluctuations in the cause of diabetic neuropathy.

In fact, recent studies disclosed that impaired vascular flow to which oxidative stress elicited from metabolic aberrations attribute[28,29] was found to be more crucial for the MNCV delay in streptozotocin-induced diabetic rats.[30] Under chronic hyperglycemic conditions, neuropathic changes may be complicated by both vascular alterations and nerve dysmetabolism. Because we did not examine nerve blood flow or peripheral nerve structure, the effects of NG treatment on the microvessels and on nerve fiber degeneration are yet to be determined. Further investigation is essential to clarify whether the current results can be applicable to the pathophysiologic basis of neuropathy in human diabetic subjects.

ACKNOWLEDGMENT

The authors thank Nozomu Ishida and Dr Akira Okano for their expert assistance.

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