Slowing of peripheral motor nerve conduction was ameliorated by aminoguanidine in streptozocin-induced diabetic rats

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Hyperglycemia is well known to accelerate the chemical formation of early glycosylation products with protein (Schiff bases and Amadori products) in diabetic subjects (1). Some of the Amadori products are changed into advanced glycosylation end-products (AGE) through a slow, complex series of chemical rearrangements. The accumulation of AGE on proteins may alter the structural and functional integrity of the macromolecules, which often play major roles in regulating cellular and vascular functions. In order to examine a causality between increased accumulation of AGE in tissue and progression of diabetic complications, it is necessary to develop specific and quantitative assay systems for the AGE. Two principally different methods have been used for determinations of AGE in biological samples: fluorescence (e.g. excitation/emission = 370/440 nm) (2) and enzyme-linked immunosorbsent assay (ELISA) with specific antibodies (3). Recently, the accumulated evidence has shown that the net increase in AGE levels of long-lived proteins in diabetic animals might be correlated with the pathological lesions of several tissues such as lens crystallins (4), renal cortex (4, 5) and aorta (6). This information came from the determination of tissue levels of AGE using an ELISA specific to the AGE. Our previous study (4) comparing two assay methods has shown that the mean AGE level in lens crystallins of diabetic rats was increased by more than 12-fold estimated by ELISA but less than two-fold by fluorescence, indicating that ELISA was more sensitive than the fluorescence level.

Although aminoguanidine (AG) is known as an inhibitor of AGE formation, the experimental evidence for this is based on several indirect observations carried out in vitro (7). There has been only one in vivo report (8) demonstrating that AG prevented AGE formation in hemoglobin of diabetic patients in a 28-day phase I clinical trial with AG, whereas there are many reports that have examined the therapeutic potential of AG in the treatment of experimental diabetic complications. Aminoguanidine ameliorated proteinuria (9, 10) and increased the thickness of the glomerular basement membrane (11). However, the mechanism whereby AG improves diabetic complications still remains obscure. There are some reports showing that AG improved slowing of motor nerve conduction velocity (MNCV) in...
diabetic peripheral nerve (12, 13). Kihara et al. showed that AG increased the microcirculation of nerve microvessels (12). On the other hand, Yagihashi et al. demonstrated an improvement of morphological changes of peripheral nerve and a decrease in AGE content estimated by levels of fluorescent AGE (13). However, so far, there has been no direct evidence in vivo demonstrating that AG ameliorates slowing of MNCV by decreasing tissue levels of AGE measured specifically by ELISA.

In this study, we have examined the effects of AG on slowing of MNCV in streptozocin-induced diabetic rats. Effects of AG on morphometric and biochemical changes in diabetic nerve and on the AGE content in renal cortex were also investigated as an indicator of AGE accumulation in vivo. The results showed that AG treatment for 16 weeks dose-dependently ameliorated conduction slowing in the sciatic nerve with a concomitant decrease in AGE content in renal cortex, suggesting that AG improved the slowing of nerve conduction through preventing the accumulation of AGE content in diabetic tissue.

Materials and methods

Chemicals

Streptozocin (STZ), 1-deoxy-1-morphorino-α-fructose, nitro blue tetrazolium, pyruvate kinase (type II) and lactate dehydrogenase were obtained from Sigma (St Louis, MO). Bovine serum albumin (BSA, fraction V, RIA grade) and keyhole limpet hemocyanin (KLH) were from Calbiochem (San Diego, CA). Horseradish peroxidase-conjugated rabbit anti-guinea pig IgG (H+L) was from Zymed Laboratories (San Francisco, CA). P-Nitrophenylphosphoric acid disodium salt was purchased from Nacalai Tesque (Kyoto, Japan). Aminoguanidine hydrochloride (AG) was synthesized from aminoguanidine bicarbonate (Regis Technologies Co., IL) at Yamanouchi Pharmaceutical Co., Ltd (Tsukuba, Japan). Other chemicals were of the best grade available from commercial sources.

Animals

Diabetes was induced by intravenous injection of streptozocin (50 mg/kg body weight) dissolved in 10 mmol/l citrate buffer (pH 4.5) in male Wistar rats of 13 weeks of age (Charles River Japan, Tokyo, Japan). After 1 week, blood glucose levels were determined by the glucose oxidase method (Autopack-A glucose, Boehringer Mannheim Yamanouchi. Tokyo, Japan). Rats displaying glucose levels of more than 16.7 mmol/l were considered diabetic and then were divided into several groups. Age-matched non-diabetic rats were used as the control. Aminoguanidine dissolved in distilled water was administered orally at doses of 10, 25 and 50 mg/kg (6 days/week) for 16 weeks. The MNCV and blood glucose were determined every 4 weeks up to 16 weeks. At the end of the experiments, all rats were sacrificed. Kidney cortex and blood were obtained for the determination of AGE and fructosamine levels. The Na⁺,K⁺-ATPase activity and morphometric examination were carried out using sciatic nerves. The experimental protocol was approved by the local animal ethics committee for animal studies.

Electrophysiology

The MNCV was measured using the method described by Sharman and Thomas (14). In brief, rats were anesthetized with sodium pentobarbital (52–65 mg/kg, ip). To minimize effects of low body temperature on the conduction velocity, the body temperature of the rat was maintained at 37–37.9°C with a thermoregulator (NP-2, Nikon, Tokyo, Japan) and heating mat. The body temperature was monitored subcutaneously with a thermometer (MGA-III, Shibaura, Tokyo, Japan). The right sciatic and tibial nerves were stimulated at the sciatic notch (proximal) and the posterior tibial nerve at the ankle (distal), respectively, by means of a monopolar needle electrode of an MS92 electromyogram device (Medelec, London, UK). Muscle action potentials were recorded from interdigital plantar muscle of the right hindlimb by using a bipolar needle electrode. The MNCV was calculated from the latency of the M-response and the distance between two stimulation points (14).

Morphometric analysis

At 16 weeks after dosing with saline or AG, rats were killed with ether. Sciatic nerves were excised rapidly and a small segment of proximal portion of the sciatic nerve was fixed in a phosphate-buffered 2.5% glutaraldehyde and 2% paraformaldehyde mixture. After embedding in epoxy resin, transverse semi-thin sections (1 μm thick) were made by an ultramicrotome (MT-2, Ivan Sorvall Inc., Norwalk, CT) and stained with toluidine blue. The mean diameter of the sciatic nerve fasciculus was measured using a micrometer. After finishing the measurement of the diameter of the sciatic nerve fasciculus of all preparations, five samples were chosen randomly from each of the groups (non-diabetic and diabetic with or without 50 mg/kg AG) in order to conduct a more detailed morphometric examination. Myelinated fiber size, diameter of myelinated fiber and myelinated fiber density were measured using an image analyzer (microcomputer system, Luzex3U, Nikon, Tokyo, Japan). Myelinated fiber size was defined as the area delineated by the outer myelin border. Myelinated fiber diameter was the length of the greatest diameter between the outer myelin borders.

Activity of Na⁺,K⁺-ATPase in sciatic nerve

About 20 mg of rat sciatic nerve was homogenized in 1 ml of Tris-HCl buffer (pH 7.5) containing 3 mmol/l of
ATP and 200 mmol/l sucrose. After removal of the pellet by brief centrifugation, aliquots of the supernatant were used for the measurement of Na\(^+\)K\(^-\) ATPase activity according to the method described by Greene et al. (15). Na\(^+\)K\(^-\) ATPase activity was defined as the rate of NADH oxidation in the absence or presence of 3 mmol/l of ouabain.

**Glycosylated serum proteins (fructosamine)**

Fructosamine was measured using the assay method described by Johnson et al. (16). Serum (0.1 ml) was incubated in 1 ml of 100 mmol/l carbonate buffer (pH 10.8) containing 0.25 mmol/l nitro blue tetrazolium at 37°C. The serum concentration of fructosamine was calculated from the difference of the absorbance at 530 nm between 10 and 15 min after the start of the incubation. 1-Deoxy-1-morphorino-o-fructose was used as a standard.

**Immunogen preparation and immunization**

Preparations of AGE-KLH and anti-AGE-KLH were reported previously (3). In brief, KLH (100 mg) was incubated with glucose (3 g) dissolved in 5 ml of phosphate-buffered saline (PBS) at 37°C for 12 weeks. After dialysis, AGE-KLH (100 µg) was emulsified in complete Freund’s adjuvant and injected intradermally into multiple sites of guinea pigs seven times at 2-week intervals. Antisera obtained 10 days after the final injection were used.

**Competitive ELISA**

The assay method was shown in detail previously (3). The AGE-BSA was prepared by the incubation of bovine serum albumin with glucose for 4 weeks. Briefly, 50 µl of AGE-BSA solution (0.5 µg/ml) in 50 mmol/l carbonate buffer (pH 9.6) was added to the wells on a 96-well multiwell plate that was then kept at 4°C overnight. After washing the plate three times with 300 µl of PBS, all wells were blocked by PBS with 5% rabbit serum for 1 h. After removal of blocking buffer followed by washing three times, 100 µl of the mixture containing 50 µl each of 1:15 000 diluted anti-AGE-KLH antisera and the samples was applied to each well on the plate, followed by incubation for 2 h. After washing the plate, 100 µl of peroxidase-conjugated rabbit anti-guinea pig IgG optimally diluted with blocking buffer was added to all wells. Two hours later, the plate was washed, 100 µl of 3.7 mmol/l o-phenylenediamine and 0.03% hydrogen peroxide solution (pH 5.0) was added. The reaction was stopped by addition of 50 µl of 0.5 mmol/l sulfuric acid and subsequently monitored at 490 nm with multichannel spectrophotometer (Model 3350 Microplate Reader, Bio-Rad Lab., Richmond, CA). Results were expressed as the ratio B/B0, calculated as experimental OD—background OD (no coating with AGE-BSA, ~0.05)/total OD (no competitor, ~0.850)—background OD. Data were expressed in arbitrary units (AU) per milligram of protein. One unit of AGE in each sample was defined as the amount of protein required to inhibit 50% of the antibody binding, and the amount of protein in each sample was used to calculate the AGE AU. At a dose of 25 ng, for example, AGE-BSA inhibited 50% of the antibody binding under our standard conditions, and the AGE unit in the AGE-BSA corresponds to 40 000 AU.

**Tissue preparation**

According to the method of Nakayama et al. (4), renal cortices (about 100 mg) from diabetic and nondiabetic were minced and washed with ice-cold PBS three times. The tissue was delipidated with chloroform and methanol (2:1) overnight. The tissue was washed again with methanol, followed by deionized water. The tissue was homogenized in 1 ml of 0.1 mmol/l sodium hydroxide and was centrifuged at 8000 g for 15 min at 4°C. An aliquot of the supernatant was subjected to ELISA for AGE. The ATPase activity was measured at 37°C with a reaction in which ATP hydrolysis was coupled to NADH oxidation, the latter being monitored at 340 nm in a spectrophotometer. Reaction mixtures were preincubated for 30 min before monitoring the linear fall in optical density over a further 10 min. Briefly, the incubation medium contained 100 mmol/l NaCl, 30 mmol/l imidazole, 10 mmol/l KCl, 3 mmol/l ATP, 2.5 mmol/l MgCl\(_2\), 0.4 mmol/l NADH, 30 µg/ml pyruvate kinase and 50 µg/ml lactate dehydrogenase, adjusting the pH to 7.3 with hydrochloric acid.

**Statistical analysis**

The MNCV data were analyzed using one-way ANOVA, followed by Student’s t-test: the pretest values and test values within one group were analyzed by a t-test for paired values. Student’s t-test were used to analyze the histomorphometric data, body weight, blood glucose and the ATPase activity. Differences were accepted as significant at the p < 0.05 level.

**Results**

**Changes in body weight and blood glucose**

Rats given STZ exhibited a marked hyperglycemia and no gain of body weight at the end of a 16-week experiment (Table 1). Aminoguanidine had no effect on blood glucose levels and body weights in diabetic rats.

**Effects of STZ-induced diabetes and AG on the MNCV**

Non-diabetic rats showed an age-related increase in the MNCV from 55.9 ± 1.1 m/s at week 0 to 72.7 ± 1.8 m/s
at week 16 (Fig. 1). However, STZ-induced diabetic rats had no increase in MNCV during the course of the experiment (57.9 ± 0.9 m/s at week 0, 56.2 ± 1.1 at week 16). Diabetic rats were treated orally for 16 weeks by AG at three doses of 10, 25 and 50 mg/kg. Twelve weeks after the start of the treatment, the MNCV in AG-treated groups was tending upward compared to untreated diabetic rats. At week 16, AG significantly increased the MNCV by 36.4%, 72.7% and 84.2% compared to the non-diabetic control in diabetic rats treated with AG at doses of 10, 25 and 50 mg/kg, respectively.

Morphometric analysis of sciatic nerve fiber from diabetic rats treated with or without AG

The diameter of the fasciculus and the means of myelinated fiber size, diameter and density of the sciatic nerves in diabetic rats were not different from those in non-diabetic rats (Table 2). There was also no apparent difference in distribution of myelinated fiber size of sciatic nerves between non-diabetic and diabetic rats (data not shown). Aminoguanidine had no effect on those parameters.

Effects of diabetes and AG on Na⁺,K⁻-ATPase activity

There was no change in composite or ouabain-inhibitable ATPase activities in sciatic nerves between non-diabetic and diabetic rats (Table 3). Aminoguanidine did not change the Na⁺,K⁻-ATPase activity that was inhibitable by ouabain.

Effects of diabetes and AG on serum fructosamine levels

About a two-fold increase in serum fructosamine level was observed in diabetic rats compared to non-diabetic rats (Fig. 2). Compatible with other reports (7, 8), AG did not cause any changes in early Amadori glycosylation products, such as serum fructosamine level.

Amounts of AGE in renal cortex from non-diabetic and diabetic rats with or without AG

To estimate the effects of diabetes and AG treatment on the tissue accumulation of AGE in vivo, the renal cortex was chosen because a specific ELISA for AGE has been established (3, 4). As shown in Fig. 3, amounts of AGE extracted by alkaline solution from renal cortex of diabetic rats showed an increase of six times over non-diabetic rats. Aminoguanidine treatment at a dose of 50 mg/kg resulted in a 40% decrease in AGE content.

Correlation of AGE content in renal cortex to MNCV in diabetic rats treated with or without AG

Figure 4 shows a significant, negative correlation between AGE content in renal cortex and MNCV in diabetic groups treated with or without AG (r = -0.55, p < 0.05). The correlation also existed among the three groups, including non-diabetic control rats (r = -0.47, p < 0.05; the line was not shown). These results suggest that a decrease in the MNCV in diabetic rats is due, at least in part, to an increase in AGE content in tissue.

Table 1. Effects of aminoguanidine (AG) administration for 16 weeks on body weight and blood glucose in streptozocin-diabetic rats.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AG (mg/kg)</th>
<th>Body weight (g) Week 0</th>
<th>Body weight (g) Week 16</th>
<th>Blood glucose (mmol/l) Week 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>0</td>
<td>492 ± 5* (15)</td>
<td>602 ± 9* (15)</td>
<td>4.85 ± 0.22*</td>
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<tr>
<td>Diabetic</td>
<td>0</td>
<td>404 ± 7 (15)</td>
<td>386 ± 15 (9)</td>
<td>24.69 ± 2.84</td>
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<tr>
<td>Diabetic</td>
<td>10</td>
<td>411 ± 5 (15)</td>
<td>384 ± 15 (11)</td>
<td>24.41 ± 1.12</td>
</tr>
<tr>
<td>Diabetic</td>
<td>25</td>
<td>427 ± 8 (15)</td>
<td>401 ± 12 (10)</td>
<td>23.57 ± 1.13</td>
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<tr>
<td>Diabetic</td>
<td>50</td>
<td>415 ± 6 (14)</td>
<td>401 ± 13 (11)</td>
<td>23.88 ± 2.87</td>
</tr>
</tbody>
</table>

*The number of observations is shown in parentheses; *p < 0.01 vs diabetic. Diabetes was induced by injection of streptozocin 1 week before week 0. Numbers of dead animals during the course of experiment are as follows: non-diabetic, 0; diabetic, 6; diabetic treated with AG, 4 (10 mg/kg), 5 (25 mg/kg) and 3 (50 mg/kg).
Diabetic pathways.

Discussion

In the present study we have shown that AG treatment for 16 weeks improved slowing of the MNCV in diabetic rats in a dose-dependent manner (Fig. 1). This occurred in the absence of any effects on body weight and blood glucose levels (Table 1), morphometric changes in the sciatic nerve (Table 2) and nerve Na⁺,K⁺-ATPase activity (Table 3). There are no lack of theories linking activated polyclonal metabolism to the progression of diabetic complications (17, 18). Activation of the polyclonal pathway affects a large variety of metabolic pathways. Several previous studies have reported that a high concentration of polyclonal inside the cell decreases the cellular uptake of myoinositol, which leads to decreased Na⁺,K⁺-ATPase activity (19). In the present study, however, ATPase activity was not decreased in diabetic nerve. Yen et al. (20) reported that Na⁺,K⁺-ATPase activity was not decreased in diabetic rat lens but the ATPase transport activity measured by ⁸⁶Rb uptake in the intact lens was decreased significantly, suggesting that the abnormality of the enzyme was attributed to the changes in the level of intracellular substrates for the ATPase rather than a decrease in the number or activity of the ATPase molecules. Further study will be needed to investigate a link between the slowing of peripheral nerve conduction and the impaired functions related to the decreased activity of Na⁺,K⁺-ATPase in diabetic peripheral nerve.

The effect of AG on the slowing of MNCV in this study (Fig. 1) was relatively delayed compared with those reported with aldose reductase inhibitors (21, 22) and an ACTH(4-9) analog (23). The aldose reductase inhibitor FR 74366 improved the slowing of diabetic nerve conduction at 2 weeks (21) and ponalrestat also showed the same effects at 4 weeks (22). In this study, AG significantly increased the MNCV at 16 weeks after AG treatment in a dose-dependent manner but was ineffective until 12 weeks (Fig. 1). This is compatible with previous reports (12, 13) demonstrating that AG did not ameliorate slowing of the MNCV until 12 or 16 weeks after the start of AG administration when the MNCV was measured serially. There was no study showing that AG ameliorated slowing of the MNCV within 8 weeks (24). The failure of quick onset of AG action, however, may not be attributed to the relatively low dose of AG used in this study because the highest dose (50 mg/kg) increased the MNCV to that of the non-diabetic level, as shown in Fig. 1 (84.2% of non-diabetic control). Low et al. (25) showed that there were at least two mechanisms of slowing of the MNCV in diabetic neuropathy. There is a myoinositol component that develops after a short period of hyperglycemia (established by 4 weeks) and a delayed hypoxic component due to a microangiopathy of endoneurial vessels. The latter component chronically reduces blood flow and impairs neural function (26). Kihara et al. reported that AG reversed nerve ischemia and improved the slowing of the MNCV by increasing the microcirculation of the nerve microvessels (12). They also showed that an increase in oxygen supplementation prevented some electrophysiological and biochemical abnormalities in experimental diabetic neuropathy (27). Odetti et al. (28) measured the time course of an increase in AGE accumulation in subcutaneous rat skin collagen estimated by the intensity of fluorescence (excitation/emission = 370/440 nm) after induction of diabetes by STZ. After a lag of 6–7 weeks, the

<table>
<thead>
<tr>
<th>Diameter of sciatic nerve fasciculus (µm)</th>
<th>Myelinated fiber size (µm²)</th>
<th>Myelinated fiber diameter (µm)</th>
<th>Myelinated fiber density (no./10² µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>789 ± 31</td>
<td>68.1 ± 3.0</td>
<td>8.7 ± 0.2</td>
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<tr>
<td>Diabetic</td>
<td>791 ± 30</td>
<td>65.9 ± 2.1</td>
<td>8.6 ± 0.1</td>
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<tr>
<td>Diabetic treated with AG</td>
<td>779 ± 20</td>
<td>62.3 ± 2.0</td>
<td>8.3 ± 0.1</td>
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</table>

* Values are means ± SEM. Number of observations is 9–10 in column 1 and 5 in columns 2–4.

Table 3. Effects of diabetes and aminoguanidine (AG) on Na⁺,K⁺-ATPase activity in sciatic nerve.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Composite</th>
<th>Ouabain-sensitive (µmol ADP·g⁻¹·wt·h⁻¹)</th>
<th>Ouabain-insensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>170.5 ± 18.7</td>
<td>82.6 ± 11.3</td>
<td>87.8 ± 10.2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>166.3 ± 19.7</td>
<td>93.4 ± 6.1</td>
<td>73.0 ± 15.2</td>
</tr>
<tr>
<td>Diabetic treated with AG</td>
<td>153.9 ± 13.9</td>
<td>73.0 ± 15.2</td>
<td>58.9 ± 12.3</td>
</tr>
</tbody>
</table>

* Rats were treated with 50 mg/kg of AG for 16 weeks. Values are expressed as means ± sxe from seven observations at study termination.
fluorescence increased in an exponential fashion until week 10 and AG prevented the increase in fluorescence. Thus, it is suggested that the onset of AG action may be dependent on the progression of diabetic neuropathy, which was influenced by the development of microangiopathy or AGE accumulation in neural tissue, or both.

Vlassara et al. (29) showed that the AGE–myelin content in peripheral nerve was increased according to the duration of diabetes. The uptake by macrophage of peripheral nerve myelin from old diabetic rats (1.5–2-

year duration) was much greater than that from the young diabetic rats with early diabetes (4–5 weeks). Furthermore, Yagihashi et al. (13) demonstrated that diabetes (16-week duration) resulted in a 3.5-fold increase in the intensity of fluorescent AGE in peripheral nerve compared to non-diabetic rats and that AG treatment at a dose of 25 mg/kg decreased the AGE content by about 35% compared to diabetic rats. The present results showed that amounts of AGE extracted by alkaline solution from renal cortex of diabetic rats were increased sixfold over non-diabetic rats (Fig. 3). The increased AGE content by diabetes was reduced by 40% on AG treatment at a dose of 50 mg/kg and also a significant. negative correlation between AGE content in renal cortex and MNCV was observed in diabetic rats treated with and without AG (Fig. 4). This finding is consistent with the observations made by Yagihashi et al. (13), suggesting that a decrease in the MNCV in diabetic rats may be due, in part, to an increase in the AGE content in nerve. Although ELISA for AGE-protein is a more specific and sensitive assay than the fluorescence assay, the numbers of tissues to which ELISA is applicable are limited in the tissues such as lens crystallins (4), renal cortex (4, 5) and aorta (6). To date, there has been no report measuring AGE content in peripheral nerve using ELISA and we have not yet established the assay method. Our preliminary data indicated that one of the causes that obstructed the application of ELISA to various tissues was the low sensitivity of the assay system. The sensitivity of the assay may be influenced by the binding affinity of antibody to AGE. AGE content in tissue and the amount of tissue collected. In order to measure the AGE content in nerve, it is necessary to use a higher affinity antibody. In the
present study, we measured AGE content in renal cortex as an indicator of AGE accumulation in the body. One of interesting findings in the present study was that the AGE content in renal cortex may be a good indicator to estimate the AGE content in the body, because a negative correlation between the AGE content in renal cortex and the MNCV was established.

Yagihashi et al. (13) demonstrated that the improvement in the slowing of conduction by AG was accompanied by a lesser reduction in myelinated fiber size and a milder axonal atrophy. However, in this study we did not observe any changes in myelinated fiber size, diameter or density in sciatic nerve of diabetic rats (Table 2). This is consistent with the early report by Sharma and Thomas, showing that the myelinated fiber population of the sural and tibial nerves in diabetic rats showed no loss of fibers or reduction in their caliber (14). We have no explanation for this difference between morphometric changes reported by Yagihashi et al. and ours. However, one possibility is the severity of the diabetic state. Although blood glucose levels were almost the same between the two studies, the recovery rate of the MNCV in diabetic rats treated with AG (25 mg/kg) in our study was double that reported by Yagihashi et al. (73% and 35% respectively). This suggests that the diabetic condition of Yagihashi’s study could be more severe than that of our study.

References