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Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals

(streptozotocin/hyperglycemia/microvascular atherogenesis/diabetic neuropathy)

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ABSTRACT **Since advanced glycosylation end products have been suggested to mediate hyperglycemia-induced microvascular atherogenesis and because aminoguanidine (AG) prevents their generation, we examined whether AG could prevent or ameliorate the physiologic and biochemical indices of streptozotocin (STZ)-induced experimental diabetic neuropathy. Four groups of adult Sprague–Dawley rats were studied: group I received STZ plus AG (25 mg·kg^{-1}·day^{-1}), group II received STZ plus AG (50 mg·kg^{-1}·day^{-1}), group III received STZ alone, and group IV was a control. We monitored conduction and action potential amplitudes serially in sciatic-tibial and caudal nerves, nerve blood flow, oxygen free radical activity (conjugated dienes and hydroperoxides), and the product of the permeability coefficient and surface area to ^{125}I-labeled albumin. STZ-induced diabetes (group III) caused a 57% reduction in nerve blood flow and in abnormal nerve conduction and amplitudes and a 60% increase in conjugated dienes. Nerve blood flow was normalized by 8 weeks with AG (groups I and II) and conduction was significantly improved, in a dose-dependent manner, by 16 and 24 weeks in sciatic-tibial and caudal nerves, respectively. The permeability coefficient was not impaired, suggesting a normal blood–nerve barrier function for albumin, and the oxygen free-radical indices were not ameliorated by AG. We suggest that AG reverses nerve ischemia and more gradually improves their electrophysiology by an action on nerve microvessels. AG may have potential in the treatment of diabetic neuropathy.**

In chronic experimental diabetic neuropathy, nerve blood flow (NBF) is reduced and the oxygen tension histogram is shifted into the hypoxic range (1). Nerve biosynthesis of 6-keto-prostaglandin $F_{1\alpha}$, the stable metabolite of prostacyclin, is significantly reduced in chronic, but not in acute, experimental diabetic neuropathy (2). Platelet thromboxane B_2 is increased (3–6), resulting in a reduced prostacyclin/thromboxane ratio and resultant vasoconstriction. Improvement in blood flow by chemical sympathectomy (7) or an increase in the oxygen supply by supplementation (8) or hyperbaric oxygenation (9) has been shown to improve nerve electrophysiology. Although these findings implicate perturbed microvascular physiology, the mechanism(s) by which chronic hyperglycemia results in a reduction in NBF is uncertain. It has been suggested that advanced glycosylation end products (AGE) may mediate hyperglycemia-induced microvascular atherogenesis (10, 11). Since aminoguanidine (AG) prevents AGE generation and has been reported to prevent basal lamina thickening in diabetic rats (10, 11), we examined whether AG could prevent or ameliorate the physiologic and biochemical indices of streptozotocin (STZ)-induced experimental diabetic neuropathy.

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METHODS

Experimental Diabetic Neuropathy. We used male Sprague–Dawley rats weighing ≈250 g. They were separated into four groups of 16 animals each: group I or AG25 received STZ plus AG (25 mg·kg^{-1}·day^{-1}), group II or AG50 received STZ plus AG (50 mg·kg^{-1}·day^{-1}), group III received STZ alone, and group IV was the control. To evaluate if AG had a separate pharmacologic vasoactive effect, two control groups were treated with AG at 25 mg·kg^{-1}·day^{-1} and 50 mg·kg^{-1}·day^{-1} for 8 weeks for NBF measurements. AG and STZ were administered by intraperitoneal injection. STZ was dissolved in 0.05 M sodium citrate at pH 4.5 (65 mg/ml; dose, 1.32 ml/kg). The control group received an intraperitoneal injection of citrate buffer alone. The animals were housed in cages with plastic floors covered with sawdust and wood shavings and were fed Purina rat chow (no. 5001) with an unrestricted supply of water. Rats were accepted as diabetic if their fasting blood glucose exceeded 16.7 mmol/liter 7 days after injection of STZ and remained >16.7 mmol/liter at the time of harvest. Electrophysiologic studies were performed on all rats. Nerve was harvested for determination of oxygen free-radical activity after which NBF was measured on the contralateral nerve. Blood–nerve barrier studies were done on separate groups of animals after electrophysiology.

Measurements of NBF. NBF was measured at 8 and 16 weeks by microelectrode hydrogen polarography (tip size, 2–5 μm) (12). The signal entered a computer (PC-XT) through an analog-to-digital converter for simultaneous display (Labtech Notebook; Laboratory Technologies, Wilmington, MA) and storage (Lotus 123; Lotus Developmental, Cambridge, MA). A curve was fitted to the data using a nonlinear regression program based on the Marquardt algorithm (13).

Electrophysiological Methods. We used techniques that are standard for our laboratory. Sensory conduction velocity and nerve action potential amplitude were measured in the caudal nerve (14). Motor conduction velocity and the amplitude of the compound muscle action potential were measured in the sciatic-tibial nerve (15). Fine stainless steel near-nerve stimulating and recording electrodes were employed. Recordings were made at 35°C, amplified 1000 times, stored on digital tape, and analyzed off-line using a Nicolet 4094 digital oscilloscope with associated stimulators and stimulus isolation units.

Serum Glucose. Serum glucose was determined using a glucose oxidase method as described (14). Measurements were obtained in duplicate at 450 nm on a LKB spectrophotometer (Ultraspec II).

Preparation of Lipid Extract. Extraction of lipid from peripheral nerve for determination of content of conjugated

Abbreviations: NBF, nerve blood flow; AGE, advanced glycosylation end products; AG, aminoguanidine; STZ, streptozotocin; CNAP, compound nerve action potential.

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dienes and hydroperoxides was achieved by the method of Folch *et al.* (16). The nerve was weighed then homogenized in 1.0 ml of ice-cold 0.9% NaCl. Two 0.4-ml samples of homogenate were transferred to 4.0 ml of chloroform/methanol, 2:1 (vol/vol). This mixture was mixed for 1 min and then centrifuged at 2500 × *g* for 10 min. The lower layer was removed for evaporation. The upper layer was washed with chloroform/methanol/H_2O, 86:14:1 (vol/vol) and centrifuged as above. The lower layers from the two steps were combined and evaporated under nitrogen. The lipids were then dissolved in 0.5 ml of chloroform/methanol and stored at −80°C until analysis.

Determination of Conjugated Dienes. Estimation of conjugated dienes was based on increases in absorbance at 233 nm, using the method of Recknagel and Ghoshal (17) and adapted for peripheral nerve. A sample of lipid extract was evaporated and redissolved in 1 ml of cyclohexane. Absorbance spectra were determined in quartz cuvettes (1-cm light path) with extraction blanks used as references. An extinction coefficient of $2.52 \times 10^4\ M^{-1}$ was used.

Determination of Lipid Hydroperoxides. Lipid hydroperoxide content was determined using the iodometric method of Buege and Aust (18). The lipid extract was dried under nitrogen and mixed with 1 ml of acetic acid/chloroform, 3:2 (vol/vol), and 50 μl of potassium iodide (1.2 g/ml) was added. The tube was capped and placed in the dark for 5 min. Cadmium acetate (3 ml of a 0.5% solution) was then added and the solution was mixed and centrifuged at 2500 rpm in a Beckman JA-20 rotor for 10 min. Absorbance of the top layer was determined at 353 nm. Lipid hydroperoxides were expressed as A_{353} units/g (wet weight) or mg of protein and compared to a standard curve (cumene hydroperoxide).

Permeability Surface Area Index to Albumin. We determined the product of the permeability coefficient and surface area to ^{125}I-labeled albumin of the blood–nerve barrier using the i.v. bolus injection technique of Ohno *et al.* (19). The blood–nerve transfer of albumin has been quantified in the endoneurium of rat sciatic nerve. The experimental details and calculations have been described in detail elsewhere (20). In brief, a brachial vein was catheterized for injection of tracers and a brachial artery was used for collecting blood samples and monitoring blood pressure. A bolus of phosphate-buffered saline containing ^{125}I-labeled rat serum albumin was injected in the brachial vein, and arterial blood was sampled for 30–45 min. Two minutes prior to sacrifice, ^{131}I-labeled albumin was intravenously administered and served as the residual plasma volume indicator.

Statistics. Statistical analysis was accomplished using unpaired 2-tailed Student's *t* test, when, as recommended by O'Brien (21), each pair-wise comparison was of interest in itself, and an analysis of variance, when no such interest existed. The effect of duration on specific indices was analyzed using regression analyses. Data were expressed as mean ± SEM. Significance was accepted at the $P < 0.05$ level.

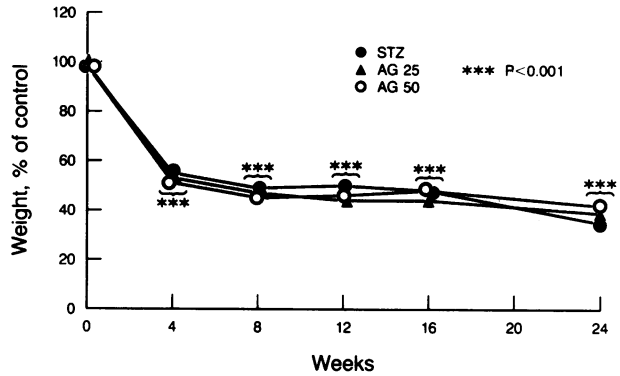

FIG. 1. Normalized mean weight, expressed as percent of controls, for each time point for the following groups: STZ, STZ plus AG at 25 mg/kg (AG25), and STZ plus AG at 50 mg/kg (AG50).

RESULTS

Weight and Blood Glucose. The mean weights of control rats at onset, 4, 8, 12, 16, and 24 weeks were 240 ± 2, 342 ± 5, 383 ± 6, 415 ± 7, 436 ± 9, and 546 ± 3 g, respectively. STZ-induced diabetic animals weighed significantly less at all time points (Fig. 1). AG hemisulfate (Sigma) treatment did not result in any weight gain. The glucose values at time of sacrifice or study were as follows: control, 5.1 ± 0.2; STZ, 27.8 ± 1.7; AG25, 27.3 ± 1.3; AG50, 26.9 ± 0.9 mmol/liter. The diabetic groups all had significantly higher glucose ($P < 0.001$) than controls and were not significantly different to one another.

NBF. The diabetic state resulted in a significant reduction ($P < 0.05$) in NBF at 8 (57%) and 16 (37%) weeks when this parameter was determined (Table 1). The reduction in flow was associated with a significant increase in microvascular nerve resistance (blood pressure/NBF) (Table 1). NBF and vascular resistance were normalized with AG treatment (Table 1). Blood pressure was greater with increasing age in both the controls and diabetic animals ($P < 0.01$ 8 weeks vs. 16 weeks). The NBF improvement was not due to a pharmacologic vasodilator effect of AG since AG treatment of control rats for 8 weeks resulted in no significant changes in NBF or microvascular nerve resistance (Table 1).

Electrophysiology. Electrophysiologic studies were done serially in both the sciatic-tibial and caudal nerves of all groups of animals.

Caudal Nerve. Caudal compound nerve action potential (CNAP) was significantly reduced in all three STZ groups (STZ, AG25, and AG50) by 8 weeks (Fig. 2). In the untreated diabetic group (STZ), CNAP was progressively more abnormal with duration of diabetes, falling to 65% and 63% of age-matched controls at 16 and 24 weeks, respectively. AG treatment resulted in improvements so that the CNAPs for AG25 and AG50 groups were 82% and 83% at 16 weeks and 88% and 97% at 24 weeks, respectively, relative to control values.

Table 1. Effect of AG on NBF, vascular resistance, and blood pressure in STZ-induced diabetic sciatic nerve

Group	8 weeks			16 weeks		
	NBF	VR	BP	NBF	VR	BP
Control	17.4 ± 3.6	8.9 ± 1.1	136 ± 5	14.7 ± 1.5	11.9 ± 1.3	165 ± 5
AG (25 mg·kg^{-1}·day^{-1})	15.7 ± 1.8	8.8 ± 1.4	158 ± 3	—	—	—
AG (50 mg·kg^{-1}·day^{-1})	14.3 ± 1.1	11.1 ± 0.7	155 ± 8	—	—	—
STZ	7.9 ± 1.5*	18.2 ± 4.3*	115 ± 5†	9.2 ± 1.5*	18.9 ± 3.4*	150 ± 4*
AG25	18.8 ± 2.9‡	6.6 ± 1.2‡	110 ± 5†	15.0 ± 0.9‡	10.0 ± 0.6§	148 ± 3*
AG50	18.2 ± 2.7‡	6.5 ± 1.2‡	103 ± 6†	14.4 ± 2.7‡	11.6 ± 1.7§	147 ± 7*

Data for NBF are expressed as ml per 100 g per min, data for vascular resistance (VR) are expressed as mm Hg per 100 mg per min, and data for blood pressure (BP) are expressed as mm Hg. $n = 8$ for each group.
*$P < 0.05$ vs. control. †$P < 0.01$ vs. control. ‡$P < 0.01$ vs. STZ. §$P < 0.05$ 8 weeks vs. 16 weeks. For BP 8 weeks vs. 16 weeks, all groups were significantly different.

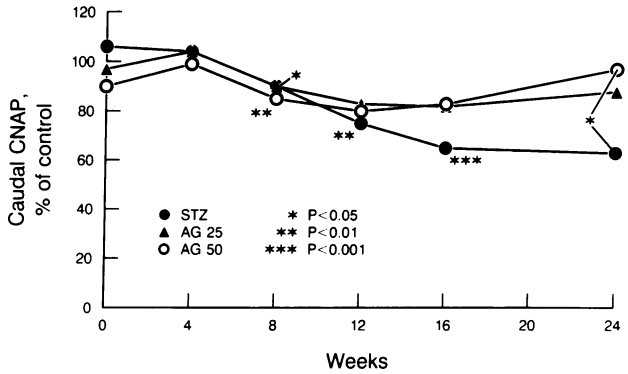

FIG. 2. Normalized mean caudal compound nerve action potential expressed as percent of control for each time point for the following groups: STZ, STZ plus AG at 25 mg/kg (AG25), and STZ plus AG at 50 mg/kg (AG50).

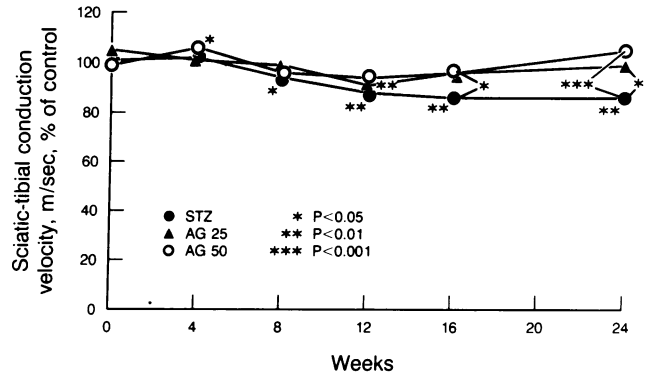

FIG. 4. Normalized mean sciatic-tibial conduction velocity expressed as percent of control for each time point for the following groups: STZ, STZ plus AG at 25 mg/kg (AG25), and STZ plus AG at 50 mg/kg (AG50).

Caudal conduction velocity (Fig. 3) was reduced by 4 weeks in the STZ group when compared with controls ($P < 0.001$) and became progressively more abnormal with increasing duration of diabetes. AG treatment resulted in a dose-dependent reversal of the slope from negative in the STZ group, to horizontal in the AG25 group, to positive in the AG50 group (Fig. 3). Dispersion was not involved since the differences between control and diabetic groups at recording distances of 2 cm and 8 cm remained unchanged (data not shown).

To further evaluate the effect of duration of treatment or disease, we compared the slopes of CNAP and conduction velocity for each animal as a function of duration (in weeks). For CNAP, the slope for the STZ group was 0.47 ± 0.51 μV/week, which was significantly reduced ($P = 0.015$) from controls (2.50 ± 0.23 μV/week). AG25 and AG50 groups exhibited slopes of 1.55 ± 0.47 and 2.45 ± 0.40, respectively, which were not significantly different from controls. The STZ group was significantly reduced when compared with the AG50 group ($P = 0.009$) but not with the AG25 group ($P = 0.14$). For conduction velocity, the results were very similar. The slope for the STZ group was -0.22 ± 0.15 m·s^{-1}·week^{-1}, which was significantly reduced ($P = 0.003$) from controls (0.49 ± 0.07 m·s^{-1}·week^{-1}). AG25 and AG50 groups yielded slopes of 0.32 ± 0.12 and 0.60 ± 0.06, respectively, and these were not significantly different from controls. The slope from the STZ group was flatter when compared with that from the AG50 group ($P = 0.001$) than with that from the AG25 group ($P = 0.022$).

Sciatic-Tibial Nerve. Sciatic-tibial nerve conduction velocity (Fig. 4) was significantly reduced in the diabetic animals by 8 weeks and became progressively more abnormal with duration of diabetes. AG treatment reduced this trend in a dose-dependent manner, so that the AG-treated nerves did not develop conduction slowing. At 4 weeks, the AG50 group exhibited conduction velocities that were 105% of controls ($P < 0.05$). By 12 weeks, AG-treated nerves responded with significantly faster conduction velocities than STZ-treated nerves (groups AG50 vs. STZ; $P < 0.05$), and both AG groups were significantly improved over untreated STZ rats at 16 and 24 weeks. At 24 weeks, the velocities for nerves from STZ, AG25, and AG50 groups were 86%, 99%, and 105% of control values, respectively.

Sciatic-tibial compound muscle action potential amplitudes were reduced in all groups at the 4-, 8-, and 12-week time points (Fig. 5). At 16 and 24 weeks, the AG50 group was not significantly different from controls. The AG25 group was significantly reduced at 16 but not at 24 weeks.

Blood–Nerve Barrier. The permeability coefficient × surface area product to albumin was unimpaired in the diabetic animals and was unaffected by AG (Table 2). No statistically significant differences were found among any of the groups (analysis of variance).

Oxygen Free Radical Activity. Sciatic nerve-conjugated diene activity was increased in all diabetic groups at the 8-week time point. AG treatment did not result in amelioration of radical activity (Table 3).

DISCUSSION

The main findings of this study are that AG administration (first measurement at 8 weeks) normalized the reduction in sciatic NBF and more gradually reversed the electrophysio-

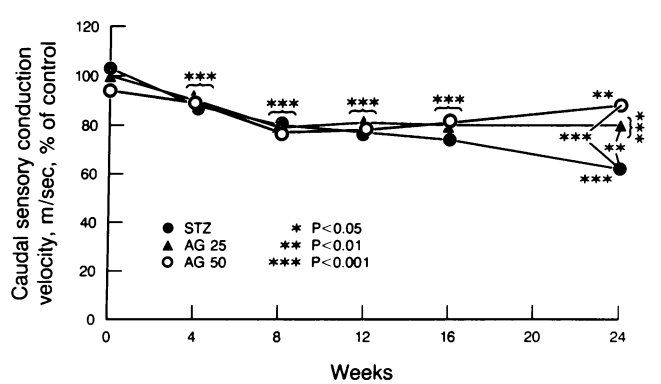

FIG. 3. Normalized mean caudal conduction velocity expressed as percent of control for each time point for the following groups: STZ, STZ plus AG at 25 mg/kg (AG25), and STZ plus AG at 50 mg/kg (AG50).

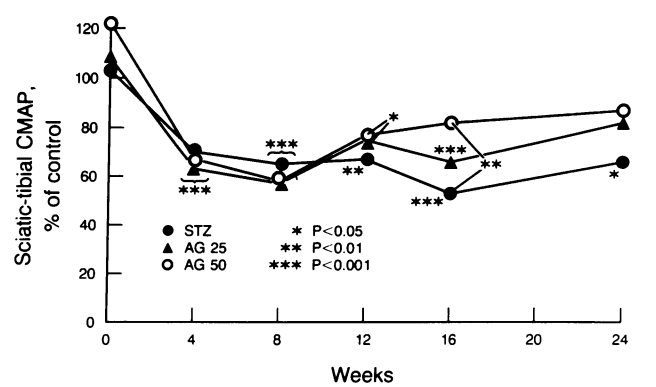

FIG. 5. Normalized mean sciatic-tibial compound muscle action potential expressed as percent of control for each time point for the following groups: STZ, STZ plus AG at 25 mg/kg (AG25), and STZ plus AG at 50 mg/kg (AG50).

Table 2. Effect of AG on the product of the diabetic sciatic nerve albumin permeability and surface area and residual endoneurial plasma volume

Group	8 weeks			16 weeks		
	n	ALB-PS	V_P	*n*	ALB-PS	V_P
Control	20	2.79 ± 0.26	1.83 ± 0.08	12	4.66 ± 0.82	2.15 ± 0.19
STZ	12	2.64 ± 0.45	1.88 ± 0.25	14	4.40 ± 0.92	1.84 ± 0.17
AG25	10	2.39 ± 0.48	2.07 ± 0.16			
AG50	12	2.86 ± 0.37	1.88 ± 0.16			

Data for the product of the albumin permeability and surface area (ALB-PS) are expressed as $ml \cdot g^{-1} \cdot s^{-1} (\times 10^{-7})$ and data for the residual endoneurial plasma volume (V_P) are expressed as $\mu l/g$.

logic abnormalities in the sciatic-tibial and caudal nerves of diabetic rats. The improvement in NBF was not due to a nonspecific vasodilator effect of AG on microvessels since AG alone did not have a vasodilator effect on normal nerves. There was no associated impairment of the blood-nerve barrier to albumin, and AG did not appear to mediate its effect through ameliorating oxygen free radical activity.

Electrophysiologic abnormalities were similar in the caudal and sciatic-tibial nerves and consisted of a progressive slowing of velocity and a reduction of the compound nerve and muscle action potentials relative to control nerves. These findings indicate that impulse transmission of the fastest conducting fibers was impaired and, since dispersion was not seen, that the number of conducting fibers was reduced. The pattern of electrophysiologic change resulting from AG administration was characteristic. Beginning 8–12 weeks after AG supplementation, there was a reversal of the slope of velocity or amplitude from negative to positive. The fact that normalization was delayed until 16 or 24 weeks in AG-treated animals and that the response was dose-dependent are suggestive of a reversal rather than a preventive effect. The abnormalities observed up to 8 weeks were essentially identical in AG-treated and untreated nerves suggesting that the neuropathy was well-established. A definitive answer to the question of reversal vs. prevention must await studies on the administration of AG after diabetic neuropathy has been established for several months.

There is a delay between the early normalization of NBF and electrophysiologic improvement. The electrophysiologic abnormalities may develop in two phases. There is an early impairment, well-established by 4 weeks, that may correspond to the metabolic neuropathy (22) and a slow progressive reduction in amplitude and velocity that corresponds in time to abnormalities observed in hypoxic nerves (9). We have demonstrated (1) that NBF is reduced and endoneurial hypoxia is present in chronic experimental diabetic neuropathy. The normalization of NBF before electrophysiologic abnormalities would be the correct sequence if the late electrophysiologic abnormalities were due to ischemia. This information could provide further support to earlier data on the prevention of electrophysiologic abnormalities with oxygen supplementation (8) and normalization of CNAP with hyperbaric oxygenation (9).

Table 3. Effect of AG on nerve conjugated dienes and hydroperoxides in STZ-treated diabetic sciatic nerve

Group	8 weeks		16 weeks	
	Conjugated dienes	Hydroperoxides	Conjugated dienes	Hydroperoxides
Control	1.8 ± 0.2	2.4 ± 0.3	2.4 ± 0.2	2.8 ± 0.3
STZ	2.9 ± 1.5†	2.6 ± 0.2	2.9 ± 0.2	2.2 ± 0.4
AG25	3.0 ± 0.2*†	2.4 ± 0.2	2.4 ± 0.3	2.4 ± 0.2
AG50	2.7 ± 0.3†	2.9 ± 0.2	2.6 ± 0.2	2.4 ± 0.4

Data for conjugated dienes and hydroperoxides are expressed as nmol/mg (wet weight).
*$n = 7$; $n = 6$ for all other groups.
†$P < 0.01$ vs. control.

Macro- and microvascular atherogenesis may be caused by the generation of AGE in chronic hyperglycemia (11). By increasing macrophage recognition and uptake, AGE stimulates macrophage-derived growth factor, which might induce smooth muscle proliferation and atherogenesis. There is also an AGE-induced increase in low density lipoproteins in vessel walls and other growth factors may be involved. There is considerable experimental support for AGE-mediated atherogenesis (11). These findings also have important treatment implications. Administration of AG results in the formation of unreactive early glycosylation products rather than AGE. Rats treated with AG for 10 months did not develop the renal glomerular basement membrane thickening seen in untreated diabetic animals (10, 11). The present study provides strong evidence for an effect of AG in ameliorating microvascular abnormalities with normalization of the increased microvascular resistance and, later, restoration of normal impulse transmission.

These improvements occurred in the absence of any increase in the product of the permeability coefficient and surface area to albumin after 8 or 16 weeks of diabetes. Our data on the blood-nerve barrier are at variance with those of another laboratory (23, 24). The studies are not directly comparable since the product of the permeability coefficient and surface area was not quantitated in the other studies. Permeability was evaluated by determining the ratio of radiolabeled albumin in nerve to a single arterial sample. Also, the investigators do not indicate whether the studies were done on endoneurium or whole nerve (24). Because NBF was reduced, it is possible that a small increase in the permeability coefficient might have been masked by a reduction in surface area. However, our study clearly demonstrates that hyperglycemia resulting in conduction slowing and reduced NBF did not cause endoneurial trapping of AGE. Instead, our data would be more consonant with the localization of AGE and AG effects on nerve microvasculature.

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