Rapid Reversal by Aminoguanidine of the Neurovascular Effects of Diabetes in Rats: Modulation by Nitric Oxide Synthase Inhibition

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Aminoguanidine treatment prevents the development of nerve conduction velocity (NCV) deficits and some renal and retinal complications in diabetic rats. Pharmacological actions include inhibition of the formation of advanced glycosylation end products (AGEs) and nitric oxide (NO) synthase. The aims of the study were to determine the extent to which diabetic NCV and nerve blood flow deficits could be corrected by aminoguanidine in an intervention study, to assess the time course of drug action, and to examine the effects of cotreatment with the NO synthase inhibitor, N^G-nitro-L-arginine (NOLA). A 19.3% ± 0.9% reduction in sciatic motor NCV after 4 weeks of untreated diabetes was corrected 86.6% ± 3.7% by aminoguanidine treatment for a further 4 weeks. Time-course studies showed that 50% of the maximal effect was attained within 6 days. Sciatic endoneurial capillary blood flow, reduced approximately 45% by diabetes, was corrected 85.6% ± 12.1% by aminoguanidine treatment. The NCV and blood flow effects of aminoguanidine were completely blocked by cotreatment with NOLA. Thus, the data support a neurovascular mechanism for aminoguanidine involving improved NO action. The rapidity of aminoguanide's effect is consistent with inhibition of free radical production by autoxidative glycosylation or glycoxidation.

ACCUMULATION of advanced glycosylation end products (AGEs) in diabetes mellitus and aging has been linked to deterioration of function in a number of organs and tissues.[1-3] Aminoguanidine traps reactive carbonyl intermediates in the Maillard reaction, reducing the synthesis of AGEs,[4,5] and treatment attenuates the development of renal, retinal, and neural abnormalities in experimental diabetes.[6-9] For peripheral nerve, aminoguanidine prevents the endoneurial perfusion deficit that is important in the pathogenesis of diabetic neuropathy.[8,10-12] Neurovascular dysfunction in diabetic rats is rapidly corrected by vasodilators, aldose reductase inhibitors, antioxidants, and ω-6 essential fatty acids.[11] However, AGEs are long-lived, and, at least in tissues such as collagen, turnover is very slow,[1-3] which has the potential to severely limit the effectiveness of aminoguanidine in intervention studies.

AGEs may alter nerve blood flow by inactivating nitric oxide (NO), thus reducing endothelium-dependent vasorelaxation.[13] Long-term aminoguanidine treatment prevents the development of deficits in acetylcholine-induced depressor responses in vivo and endothelium-dependent relaxation in vitro in diabetic rats.[13,14] However, aminoguanidine may also act "downstream" of the vascular system, preventing any functional effects of AGE formation on structural proteins in neurons and Schwann cells.[15] Furthermore, aminoguanidine can inhibit NO synthase, a phenomenon suggested to provide an alternative explanation for the beneficial effects of long-term treatment on diabetic complications.[16,17] In contrast, conventional NO synthase inhibitors such as N^G-nitro-L-arginine (NOLA) have deleterious effects on nerve function in nondiabetic and diabetic rats, caused by a reduction in endoneurial perfusion.[18,19] Thus, to further define aminoguanidine's action in experimental diabetic neuropathy, the present experiments examined whether treatment could reverse established deficits in nerve perfusion and conduction and investigated the effects of joint aminoguanidine and NOLA treatment.

MATERIALS AND METHODS

Experimental Groups, Diabetes Induction, and Treatment

Male Sprague-Dawley rats (Aberdeen University breeding colony) aged 19 weeks at the start of the study were used. Nondiabetic rats acted as onset controls. Diabetes was induced by streptozotocin (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) freshly dissolved in sterile 0.9% saline (40 to 45 mg · kg^{-1} by intraperitoneal injection) and verified 24 hours later by the presence of hyperglycemia and glucosuria (Visidex II and Diastix; Ames, Slough, UK) in nonfasted rats. After final experiments, plasma glucose was estimated (GOD-Perid method; Boehringer, Mannheim, Germany) in samples taken from the carotid artery cannula or by cardiac puncture (time-course experiments).

After 4 weeks of untreated diabetes, groups of rats were treated for another 4 weeks with aminoguanidine (Sigma, Poole, Dorset, UK) dissolved in the drinking water such that the dose was approximately 1.0 g · kg^{-1} · d^{-1}. Previous investigations have shown that this treatment regimen prevents the development of nerve conduction velocity (NCV) and renal effects (proteinuria and mesangial expansion) in diabetic rats.[6,9] A further group of aminoguanidine-treated diabetic rats were also given the NO synthase inhibitor, NOLA (Sigma), at a dose of approximately 10 mg · kg^{-1} · d^{-1} in the drinking water to examine whether aminoguanidine's effects on nerve function occurred downstream of any effects on blood flow. This dose produces a moderate level of NO synthase blockade, which has modest neurovascular effects in nondiabetic and diabetic rats.[18] Separate groups of rats were used to determine the time course of reversal of NCV changes. The initial duration of untreated diabetes was 7 weeks, followed by aminoguanidine treatment for 3 to 12 days.

Sciatic Motor NCV and Endoneurial Blood Flow

Rats were anesthetized with thiobutabarbital sodium (Zeneca) by intraperitoneal injection (50 to 100 mg · kg^{-1}). The trachea was cannulated for artificial respiration and a carotid cannula was used to monitor blood pressure. Body core and nerve temperatures were kept in the range 37° to 38°C. Motor NCV to tibialis anterior muscle was measured between the sciatic notch and the knee as previously described.[9]

Endoneurial blood flow was measured in the contralateral limb by microelectrode polarography and hydrogen clearance as previ-

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ously described.[20] Rats underwent neuromuscular blockade with D-Tubocurarine (Sigma; 2 mg · kg^{-1} via the carotid cannula) and were artificially ventilated. The level of anesthesia was monitored by observing any reaction of blood pressure to manipulation, and supplementary anesthetic was administered as necessary. A glass-insulated H_2-sensitive platinum microelectrode was inserted into the middle portion of the sciatic nerve. H_2 (10%) was added to the inspired gas, with the proportions of O_2 and N_2 being adjusted to 20% and 70%, respectively. When the H_2 current recorded by the electrode had stabilized, the H_2 supply was shut off and the clearance curve monitored. This was repeated at another nerve site. Monoexponential or biexponential curves were fitted to the data by regression (Inplot; Graphpad, San Diego, CA). The slow exponent was taken to reflect nutritive flow, and the weighted composite sum of both exponents, total (capillary, large-vessel, and arteriovenous shunt) endoneurial flow.[20,21] Vascular conductance was calculated by dividing blood flow by mean arterial blood pressure during the recording period.

Statistical Analysis

The data were subjected to Bartlett's test for homogeneity of variances and normalized by log transformation if necessary (blood flow measurements). One-way ANOVA was performed, followed by the Student-Newman-Keuls test to estimate the significance of differences for individual between-group comparisons. Kruskal-Wallis nonparametric ANOVA followed by Dunn's test was used for blood pressure data. P less than .05 was considered statistically significant. The data are expressed as the mean ± SEM.

RESULTS

Diabetes caused a 5.5-fold elevation of plasma glucose (Table 1). Rats lost weight (~36%) over the 8-week period, most of which (~28%) occurred within the first month. These parameters were not affected by aminoguanidine or NOLA treatment during the second month. Motor NCV (Fig 1) was reduced by 19.3% ± 0.9% after both 4 and 8 weeks of diabetes ($P < .001$). Aminoguanidine treatment corrected the diabetic NCV deficit by 86.6% ± 3.7% ($P < .001$), although the resultant value remained significantly ($P < .05$) less than that of the nondiabetic control group. NOLA cotreatment completely prevented ($P < .001$) the effect of aminoguanidine such that NCV was not significantly different from the values in 4- or 8-week diabetic groups.

The time course for reversal of motor NCV deficits by aminoguanidine treatment after 7 weeks of untreated diabetes was measured in four further groups (n = 6 to 7). Body weight was reduced by 33.8% ± 1.5% to 313 ± 7 g, and plasma glucose concentration was 43.3 ± 0.8 mmol · L^{-1}. NCV (Fig 2) was not significantly altered by 3 days of aminoguanidine treatment; however, after 6 days there was a 46.0% ± 4.5% ($P < .001$) correction of the diabetic deficit. After 12 days, NCV (61.58 ± 0.44 m · s^{-1}) had reached asymptote and was almost identical to that seen for 4 weeks of treatment (61.58 ± 0.45 m · s^{-1}; Fig 1).

Nutritive endoneurial blood flow (Fig 3A) was reduced 44.3% ± 4.2% ($P < .001$) after 4 weeks of diabetes, and this was sustained at 8 weeks (45.8% ± 2.7% deficit, $P < .001$). Aminoguanidine treatment during the second month reversed the diabetic blood flow deficit ($P < .001$) by 85.6% ± 12.1%; the resultant value was not significantly different from that of nondiabetic rats. In diabetic rats treated jointly with aminoguanidine and NOLA, the flow was not significantly different from that of the untreated diabetic groups, and was reduced ($P < .001$) compared with aminoguanidine treatment alone. Mean systemic blood pressure (Fig 3B) tended to be reduced by diabetes but it did not reach statistical significance. Blood pressure was unaffected by aminoguanidine, but was elevated by NOLA cotreatment ($P < .01$). To take into account perfusion pressure differences, the data are expressed as endoneurial nutritive vascular conductance (Fig 3C). The approximately 38% reduction in conductance ($P < .001$) for untreated

Table 1. Body Weight, Plasma Glucose Concentration, and Composite Blood Flow and Vascular Conductance

Group	No.	Body Weight (g)	Plasma Glucose (mmol · L^{-1})	Composite Blood Flow (mL · min^{-1} · 100g^{-1})	Composite Vascular Conductance (mL · min^{-1} · mmmHg^{-1})
Nondiabetic	10	464 ± 10	7.5 ± 0.4	68.2 ± 10.7	0.49 ± 0.07
1-month diabetic	10	335 ± 9	39.1 ± 1.6	24.7 ± 2.8*	0.21 ± 0.02*
2-month diabetic	10	288 ± 13	40.1 ± 1.9	22.5 ± 1.5*	0.19 ± 0.01*
Aminoguanidine reversal	12	303 ± 10	42.7 ± 1.2	30.1 ± 4.1*	0.27 ± 0.03*
Aminoguanidine + NOLA	7	279 ± 10	42.9 ± 1.8	25.4 ± 5.2*	0.18 ± 0.03*

NOTE. Results are the mean ± SEM.
*$P < .001$ *v* nondiabetic.

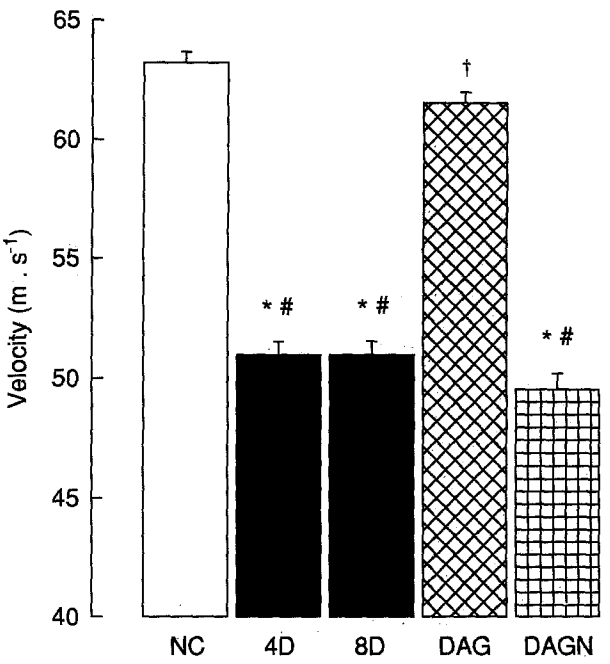

Fig 1. Effects of diabetes, aminoguanidine, and aminoguanidine + NOLA treatment on sciatic motor NCV. NC, nondiabetic control group, n = 10; 4D, 4-week diabetic group, n = 10; 8D, 8-week diabetic group, n = 10; DAG, 8-week diabetic group untreated for the first 4 weeks and then treated with aminoguanidine (1g · kg^{-1} · d^{-1}) for 4 weeks, n = 13; DAGN, 8-week diabetic group treated jointly with aminoguanidine and NOLA (10 mg · kg^{-1} · d^{-1}) during the final 4 weeks. Results are the mean ± SEM. *$P < .001$ *v* NC; †$P < .05$ *v* NC; #$P < .001$ *v* DAG.

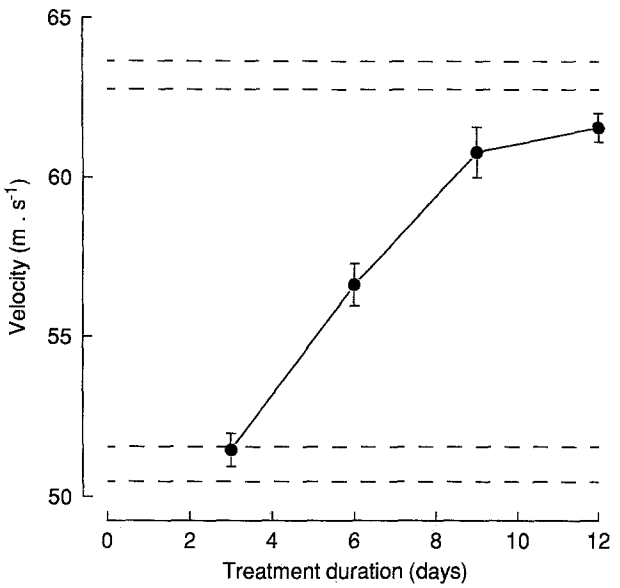

Fig 2. Time course of reversal of sciatic motor NCV deficits by aminoguanidine treatment in diabetic rats. Groups (n = 6 to 7) of 7-week diabetic rats were treated with aminoguanidine (1 g · kg^{-1} · d^{-1}) for 3, 6, 9, or 12 days before NCV measurement. (---) Envelopes of the mean ± SEM for nondiabetic and 8-week untreated diabetic groups from Fig 1 for reference.

diabetes was completely (133.2% ± 13.8%, $P < .001$) corrected by aminoguanidine treatment, although a trend toward supernormal conductance was not statistically significant. NOLA cotreatment prevented ($P < .001$) the aminoguanidine-induced increase in conductance, which was within the lower half of the untreated-diabetic range.

Diabetes also depressed total (composite) endoneurial blood flow (Table 1) by 63.8% ± 4.1% after 4 weeks and 67.0% ± 2.2% after 8 weeks ($P < .001$). Corresponding values for the deficits in vascular conductance were 57.1% ± 4.1% and 61.2% ± 2.0%, respectively. Reversal aminoguanidine treatment did not significantly affect these parameters (55.9% ± 6.0% flow and 44.9% ± 6.1% conductance deficits *v* the nondiabetic group). The combined aminoguanidine and NOLA–treated group had composite flow and conductance values within the untreated diabetic range.

DISCUSSION

The data show that aminoguanidine rapidly corrects NCV deficits in diabetic rats. The 50% NCV correction time for antioxidant[22] or aldose reductase inhibitor (K.C. Dines, M.A. Cotter, and N.E. Cameron, unpublished observations, November 1994) treatments is 5.8 and 6.4 days, respectively. In comparison, the correction time was twice as fast for the vasodilator, lisinopril (3.2 days), and the prostanoid precursor, evening primrose oil (2.7 days).[23,24] The comparable time course for aminoguanidine, antioxidant, and aldose reductase inhibitors could indirectly suggest similar mechanisms of neurovascular action.[11] Rapid reversal is possible in short-term diabetes because nerve fiber damage is modest and conduction defects depend on pathophysiological rather than gross morphological changes.[11,25] Treatment effects in rats may be mechanistically similar to those causing a relatively rapid partial improvement in patients using vasodilators,[26] for example. Previous aminoguanidine nerve studies were preventive; Kihara et al[8] showed increased NCV after an initial 16-week delay, and Yagihashi et al[27] observed an effect over 8 weeks accompanied by partial prevention of axon dwindling. These studies were performed in a young rat model in which the deficit relative to age-matched nondiabetic controls results in part from blunted nerve fiber growth[28] that develops over 4 to 8 weeks.[8] In addition, NCV was mea-

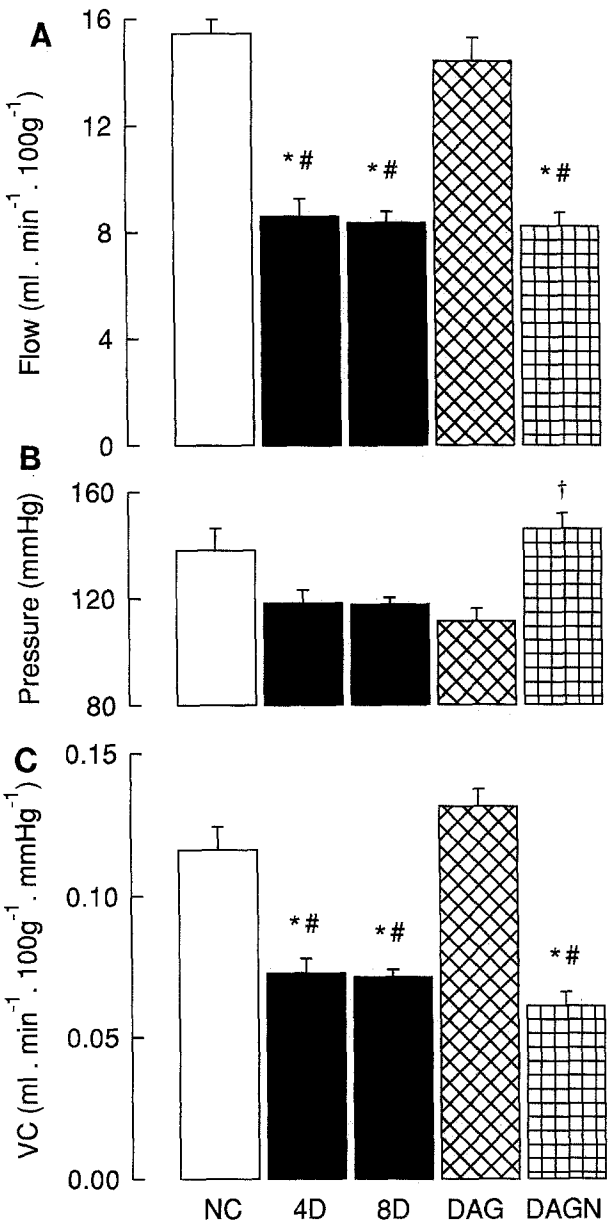

Fig 3. Effects of diabetes, aminoguanidine, and aminoguanidine + NOLA treatment on (A) sciatic nutritive endoneurial blood flow, (B) mean systemic blood pressure, and (C) nutritive endoneurial vascular conductance (VC). Groups are defined in Fig 1. Results are the mean ± SEM. *$P < .001$ *v* NC; #$P < .001$ *v* DAG; †$P < .01$ *v* DAG.

sured in relatively slowly conducting nerve branches, and aminoguanidine doses were at least 1 order of magnitude lower than in this study. One or more of these factors could explain the discrepant time course of NCV effects as compared with the results of this and a previous[9] study using mature rats. In the latter model, nerve growth is modest, NCV defects are relative to the starting value at diabetes induction, and the rapidly conducting fibers most affected by diabetes[29] were monitored. Under those conditions, high-dose aminoguanidine completely prevented motor and sensory NCV deficits over 8 weeks,[9] in agreement with data from this intervention study.

Electrophysiological effects of aminoguanidine were accompanied by correction of reduced endoneurial nutritive blood flow, in agreement with the findings of Kihara et al[8] using a prevention paradigm. However, aminoguanidine did not correct reduced composite endoneurial flow. This suggests that perfusion of the endoneurial capillary bed was elevated without a parallel increase in arteriovenous shunt flow. A selective increase in nutritive perfusion was also noted for aldose reductase inhibitor treatment.[30] One investigation found no effect of aminoguanidine on nerve laser-Doppler flux in diabetic rats.[31] However, that method monitors total red blood cell flux, biased toward the epineurium/perineurium rather than endoneurium.[10,32] Although the various blood flow compartments are linked in the highly anastomotic vasa nervorum,[33] laser-Doppler fluximetry would not necessarily have sufficient resolution to detect changes specific to the endoneurial capillary bed.[34,35]

Aminoguanidine did not significantly affect arterial blood pressure. This contrasts with a report that a dose of 400 $\mu mol \cdot kg^{-1}$, approximately 10-fold less than used in this study, caused a blood pressure elevation comparable to that induced by a high dose of the NO synthase inhibitor, N^G-monomethyl-L-arginine.[16] No pressor effects of lower doses of aminoguanidine were noted in previous studies.[8,13] Thus, it is unlikely that aminoguanidine inhibits endothelial constitutive NO synthase in vivo, a relative specificity for the inducible isoform being noted in vitro.[16,36] One report claimed that aminoguanidine acutely inhibits acetylcholine-stimulated NO-mediated endothelium-dependent relaxation of aortas in vitro.[17] However, we did not observe any inhibitory action in this preparation,[14] and no effect was seen for pulmonary arteries except with exposure to bacterial endotoxin to stimulate inducible NO synthase.[36] Furthermore, long-term aminoguanidine treatment prevents the development of impaired aorta endothelium-dependent relaxation in diabetic rats.[14] Thus, aminoguanidine's beneficial action in diabetes is unlikely to involve NO synthase blockade, and probably depends on AGE inhibition.

NOLA completely blocked aminoguanidine effects on NCV and blood flow, providing further evidence against the hypothesis of NO synthase inhibition in aminoguanidine action.[16] In nondiabetic rats, chronic NO synthase blockade causes NCV deficits, although the NOLA dose used in this investigation only produces relatively modest abnormalities.[18-20] NO synthase inhibition also abolished aldose reductase inhibitor and antioxidant actions on NCV and perfusion in diabetic rats.[19,20,37] This evidence supports a causal link between vascular and NCV changes and suggests that aminoguanidine may improve vasa nervorum endothelial NO output or action, which is depressed by diabetes.[11,38] However, the effect of NOLA alone does not unequivocally establish a NO-related mechanism, because of response nonlinearities due to interactions between local vasoactive systems, including prostanoid metabolism, that degrade functional specificity for single-inhibitor studies.[18,20] Nevertheless, AGEs inactivate NO, blocking functional effects in cell culture.[13,39] Moreover, in vivo aminoguanidine attenuates defective depressor responses to intraarterial acetylcholine in diabetic rats.[13] Thus, aminoguanidine may prevent NO destruction, which could account for the increased nutritive endoneurial perfusion. Diabetic NCV and blood flow deficits are partially prevented by NO donor treatment.[40] Aminoguanidine's effect on NCV was inhibited by NOLA, suggesting that a direct neuronal action to correct AGE-induced alterations in axon cytoskeletal proteins is relatively unimportant in acute diabetes, although a long-term effect against axon dwindling cannot be ruled out.[15,28]

Aminoguanidine's neurovascular action may depend on preventing AGE accumulation in subendothelial collagen, which could quench NO as it diffuses to smooth muscle.[13] However, the endoneurial nutritive blood flow deficit reaches 90% of its nadir within 7 days of diabetes induction,[41] which is rapid compared with the 1 to 2 months of diabetes necessary for AGE accumulation sufficient to diminish endothelium-dependent vasodilation.[13] Collagen AGE turnover is very slow,[1,2] which precludes an AGE/NO-quenching explanation for aminoguanidine's rapid NCV effect. AGE formation is prevented by the binding of aminoguanidine to reactive dicarbonyl Maillard-reaction intermediates.[4,5] In the presence of oxygen, these compounds normally generate reactive oxygen species, catalyzed by transition metal ions.[1,5] Thus, although aminoguanidine is not an antioxidant or metal chelator,[5] inhibition of autoxidative glycosylation would eliminate a free radical source in diabetes.[42] This could explain aminoguanidine's rapid neurovascular action, comparable to antioxidants and chelators,[22,37,43] because effects would depend on reducing glycosylation flux rather than simply preventing AGE accumulation. The result would be protection against free radical action on the vasa nervorum endothelial NO system.[14] Aminoguanidine did not alter the increased conjugated-diene content of sciatic nerve,[8] a marker of lipid peroxidation; however, it prevented AGE-related low-density lipoprotein oxidation.[44,45] Oxidized low-density lipoprotein is cytotoxic to endothelial cells and reduces NO-mediated vasorelaxation.[46,47] A further possibility concerns rapid intracellular glycosylation caused by highly reactive glucose metabolites,[2] which may alter the function of enzymes such as NO synthase. In addition, aminoguanidine improved decreased red blood cell deformability in diabetic rabbits, which could aid tissue perfusion.[48] However, the significance of this potential rheological mechanism for nerve is unclear. Nerve capillaries have a large diameter,

approximately twice that in skeletal muscle,[49] which minimizes the occurrence of deformation when red blood cells traverse vasa nervorum.

In conclusion, aminoguanidine treatment rapidly corrects nerve conduction and perfusion deficits in experimental diabetes. The data support a vascular view of the etiology of diabetic neuropathy compatible with the notion that reduction of free radical effects on endothelial NO-mediated relaxation by suppression of autoxidative glycosylation could account for aminoguanidine's beneficial action.

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