Only limited effects of aminoguanidine treatment on peripheral nerve function, (Na⁺,K⁺)-ATPase activity and thrombomodulin expression in streptozotocin-induced diabetic rats

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Abstract

Aims/hypothesis. Aminoguanidine, a potent anti-glycation reagent, is known to be beneficial in experimental diabetic neuropathy. In this study, we explored the mechanisms of how aminoguanidine inhibits neuropathic changes in diabetes and compared its effects with those of insulin treatment.

Methods. Wistar rats, aged 8 weeks, were made diabetic by streptozotocin and given aminoguanidine dissolved in drinking water (1 g/l) for 8 weeks. Effects of daily insulin (protamine-zinc) treatment were also examined for comparison. At the end of the 8 weeks, we examined the peripheral nerve function and (Na⁺,K⁺)-ATPase activity and their relation to serum thrombomodulin concentrations that are considered as a marker of endothelial injury.

Results. Aminoguanidine treatment reduced the diabetes-induced decrease in tibial nerve conduction velocity by 47% ($p < 0.05$ vs untreated diabetic rats)

and inhibited the loss of sciatic nerve (Na⁺,K⁺)-ATPase activity by 54% ($p < 0.05$ vs untreated diabetic rats). Insulin-treatment of diabetic rats restored these variables by 83% and 75%, respectively (both, $p < 0.01$ vs untreated diabetic rats). Thrombomodulin concentrations were increased ($p < 0.01$) in diabetic rats compared with those in non-diabetic controls and unaffected by aminoguanidine treatment. In contrast, the concentrations remained within the normal range in the insulin-treated group.

Conclusion/interpretation. Although aminoguanidine treatment improved nerve conduction velocity and (Na⁺,K⁺)-ATPase activity, its effects were considerably less than those of insulin and were not apparent in some measures of endothelial cell injury. [Diabetologia (1999) 42: 743–747]

Keywords Experimental diabetic neuropathy, aminoguanidine, insulin, nerve conduction velocity, (Na⁺,K⁺)-ATPase activity, thrombomodulin Introduction.

Inhibition of glycation by use of anti-glycation agents like aminoguanidine is now regarded as a promising new way for the prevention and treatment of vascular and neuropathic complications in diabetes [1, 2]. Several laboratories have reported beneficial effects of

aminoguanidine on peripheral nerve function in experimental models of diabetic neuropathy [3–5]. In these studies, the aminoguanidine effects were accompanied by an improvement in nerve blood flow [3, 4] and reduction of advanced glycation end product (AGE) formation in nerve tissues [5]. It has also been shown that aminoguanidine treatment can prevent the structural changes of endoneurial microvessels in long-term diabetic rats [6]. The mechanisms for these effects of aminoguanidine and the relation between endothelial cell injury and peripheral nerve dysfunction, however, are not clearly understood.

Thrombomodulin, a high-affinity receptor for thrombin, is a membrane glycoprotein found on the surface of endothelial cells [7, 8]. Increased plasma

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Abbreviations: AGE, Advanced glycation end product; STZ, streptozotocin; MNCV, motor nerve conduction velocity; FWCV, F-wave conduction velocity; NO, nitric oxide.

Table 1. Laboratory data of experimental rats and serum thrombomodulin concentrations

Group	<i>n</i>	Body weight (g)	Fasting blood glucose (mmol/l)	Glycated haemoglobin (%)	Thrombomodulin (ng/ml)
Control	6	395 ± 6	4.1 ± 0.3	1.71 ± 0.20	395 ± 24
Diabetic	6	268 ± 21 ^a	20.9 ± 4.3 ^a	3.39 ± 0.32 ^a	472 ± 28 ^a
Diabetic+aminoguanidine	6	262 ± 23 ^a	21.8 ± 3.6 ^a	3.13 ± 0.27 ^a	481 ± 71 ^a
Diabetic+insulin	7	345 ± 20 ^{a, b}	6.8 ± 0.5 ^{a, b}	1.79 ± 0.32 ^b	404 ± 25 ^b

Values are means ± SD. ^a *p* < 0.01 vs control, ^b *p* < 0.01 vs diabetic and diabetic+aminoguanidine

thrombomodulin is thought to reflect its release from cell membranes as a result of endothelial damage [9, 10]. In diabetic patients, raised thrombomodulin concentrations in the blood were accompanied by microangiopathic complications [11, 12], suggesting that they reflect endothelial injury and predict the development of microangiopathy [11, 12]. Although it has been suggested that aminoguanidine can inhibit the development of microvascular complications in diabetic animal models [1], effects of aminoguanidine on the endothelial damage has never been addressed *in vivo*.

To explore the mechanisms of aminoguanidine effects on experimental diabetic neuropathy, we examined peripheral nerve function and (Na⁺,K⁺)-ATPase activity in aminoguanidine-treated diabetic rats and compared them with those in the insulin-treated group. We also quantified serum thrombomodulin concentrations as a measure of endothelial cell damage to determine whether the benefits of treatment with aminoguanidine are related to the ability to prevent endothelial cell injury.

Materials and methods

Male Wistar rats (Japan Clea, Tokyo, Japan) aged 8 weeks were made diabetic with an intravenous injection of streptozotocin (STZ) (40 mg/kg; Sigma Chemicals, St Louis, Mo., USA) dissolved in 0.1 mol/l citric buffer (pH 4.2). Diabetic rats with fasting blood glucose concentrations more than 16.7 mmol/l were randomly separated into three groups with six to seven animals per group 1 week after STZ injection. The first group was treated for 8 weeks with aminoguanidine (Wako Pure Chemical Industries, Osaka, Japan) given in the drinking water (1 g/l), yielding a daily dose of about 370 mg/kg body weight. The second group was treated with protamine-zinc insulin (PZI) (Takeda Chem., Osaka, Japan) given as a single dose (1400 hours, s.c.) ranging between 0 and 6 units/kg, adjusted to the grade of glycosuria and glucose concentrations, measured with plastic sticks (Diasticks II, Miles-Sankyo, Tokyo). By this method, the fasting blood glucose concentrations were maintained between 4.5 and 11 mmol/l. The last group of diabetic animals remained untreated for 8 weeks. Age-matched and sex-matched normal Wistar rats served as controls.

At the end of the experiment, motor nerve conduction velocity (MNCV) of the tibial nerve between the ankle and knee, F-wave latency (FWL) and F-wave conduction velocity (FWCV) at the ankle were measured as described previously [13]. Blood glucose concentrations were measured using the glucose-oxidase method (To-Echo Super II, Kyoto Daiichi Ka-

gaku, Kyoto, Japan) and glycated haemoglobin values were measured with a microassay (HbA_{1c} Columntest; Bio-Rad, Richmond, Calif., USA). Serum thrombomodulin concentrations were quantified with an enzyme immunoassay (Mitsubishi Gas Chemical, Niigata, Japan).

Sciatic nerve (Na⁺,K⁺)-ATPase was measured in samples from all of the experimental groups. For the measurements of (Na⁺,K⁺)-ATPase activity, the perineurium was removed from sciatic nerves and the activity was determined by the spectrophotometric enzymatic method as described [14]. The activity of ouabain-sensitive (Na⁺,K⁺)-ATPase was calculated by the difference between ATPase activity with or without 1 mmol/l ouabain.

Immunohistochemical staining for thrombomodulin was done on paraffin sections of the sciatic nerve fixed with the AMeX method [15], using the streptavidin biotin complex method (Histofine SAB-PO Kit) (Nichirei, Tokyo, Japan). In brief, deparaffinized 4 µm-thick sections were incubated with rabbit anti-rat thrombomodulin antibody (1:2000 dilution) (Mitsubishi Gas Chemical) overnight at 4°C. The sections were subsequently processed with a biotinylated anti-rabbit antibody for 1 h at room temperature and with streptavidin-peroxidase complex for an additional hour at room temperature. The peroxidase-conjugated antigens were made visible with 3,3'-diaminobenzidine hydrochloride and the sections were finally counterstained with haematoxylin.

All group values were expressed as means ± SD. Statistical analysis was by a one-way analysis of variance with the Bonferroni correction for multiple comparisons, using StatView 4.0 (Abacus Concepts, Berkeley, Calif., USA). A *p* value of less than 0.05 was considered statistically significant.

Results

In samples collected at the end of the experiment, blood glucose concentrations and glycated haemoglobin values were increased in untreated diabetic rats compared with non-diabetic controls (*p* < 0.01). The body weights of the untreated diabetic rats were about 67% of the non-diabetic control animals (Table 1). Daily water consumption of aminoguanidine-treated diabetic rats was approximately 370 ml · kg⁻¹ · day⁻¹, producing an average daily aminoguanidine dose of 97 mg · day⁻¹ · rat⁻¹. Treatment with aminoguanidine had no effect on body weight, blood glucose concentrations or HbA_{1c}. In contrast, these variables were nearly normal in the insulin-treated diabetic rats (Table 1).

Serum thrombomodulin concentrations were increased (*p* < 0.01) in diabetic rats compared with the normal control rats (Table 1). Whereas treatment

Table 2. Effects of aminoguanidine and insulin treatment on peripheral nerve function and (Na⁺, K⁺)-ATPase activity in experimental rats

Group	n	MNCV (m/sec)	FWL (m/sec)	FWCV (m/sec)	(Na ⁺ , K ⁺)-ATPase activity (nmol ADP · mg ⁻¹ · h ⁻¹)	
					Composite	Ouabain-sensitive
Control	6	59.2 ± 5.2	7.0 ± 0.3	39.1 ± 2.0	325 ± 64	72.5 ± 13.4
Diabetic	6	44.2 ± 5.0 ^a	7.4 ± 0.2	34.4 ± 0.3 ^a	269 ± 53	30.9 ± 12.8 ^a
Diabetic+aminoguanidine	6	51.3 ± 3.8 ^b	7.1 ± 0.3	37.7 ± 2.2 ^b	281 ± 45	53.2 ± 14.8 ^b
Diabetic+insulin	7	56.7 ± 3.4 ^{c, d}	7.2 ± 0.2	38.0 ± 2.1 ^c	377 ± 69	62.3 ± 12.7 ^c

Values are means ± SD. ^a $p < 0.01$ vs control, ^b $p < 0.05$ vs diabetic, ^c $p < 0.01$ vs diabetic, ^d $p < 0.05$ vs diabetic + aminoguanidine

with aminoguanidine had no effect, insulin-treatment maintained thrombomodulin concentrations within the normal range.

With immunostaining thrombomodulin expression was localized to endothelial cells of perineurial and endoneurial vessels in the sciatic nerve (not shown). Endothelial cells in other organs expressed positive reactions on the luminal surface of the vascular lumen, predominantly of middle-sized arteries and capillaries. There were some weakly stained capillaries of endoneurial microvessels in untreated diabetic rats. It was, however, difficult to quantify the differences of intensities of thrombomodulin immunoreactions due to the small number of vessels among all the experimental rat groups (not shown).

Motor nerve conduction velocity and FWCV were decreased ($p < 0.01$) in untreated diabetic rats by 25 % and 12 %, respectively compared with those in non-diabetic control rats (Table 2). Treatment with aminoguanidine reduced the decrease of MNCV and FWCV by 47 % and 70 % respectively, in diabetic rats ($p < 0.05$ for both vs untreated diabetic rats). Insulin treatment also reduced the decrease of MNCV and FWCV by 83 % and 76 %, respectively ($p < 0.01$ for both vs untreated diabetic rats). The mean MNCV in the insulin-treated group was greater than that in the aminoguanidine-treated group ($p < 0.05$). There were no statistically significant differences in mean values of MNCV and FWCV between aminoguanidine-treated diabetic rats and non-diabetic controls. There was no statistically significant change in FWL among all experimental groups.

Ouabain-sensitive (Na⁺, K⁺)-ATPase activity in the untreated diabetic rats was reduced to approximately 43 % of that in non-diabetic control animals ($p < 0.01$). Treatment with aminoguanidine reduced this loss by 54 % ($p < 0.05$ vs untreated diabetic rats) whereas the insulin-treated group showed 75 % inhibition of the decrease in (Na⁺, K⁺)-ATPase activity. There was no statistically significant difference between aminoguanidine-treated diabetic rats and non-diabetic control rats. The ouabain-resistant fraction of (Na⁺, K⁺)-ATPase activity did not differ among the experimental groups.

Discussion

Our study showed that aminoguanidine treatment for 8 weeks partially protected (Na⁺, K⁺)-ATPase activity by 54 % and peripheral nerve function in diabetic rats, showing 47 % recovery of MNCV and 70 % of FWCV compared with those in untreated diabetic rats without affecting raised serum thrombomodulin concentrations. Given that these raised concentrations non-specifically reflect endothelial injury in various vascular disorders [9–12], the ameliorative effects of aminoguanidine treatment on peripheral nerve function in diabetic rats may not be dependent on the prevention of systemic endothelial injury. In contrast to the effects of aminoguanidine, daily insulin treatment further prevented the decrease in MNCV, FWCV and (Na⁺, K⁺)-ATPase activity in diabetic rats to 96 %, 97 % and 86 % of that found in non-diabetic controls, while simultaneously maintaining normal serum thrombomodulin concentrations. Treatment with aminoguanidine at this dose and duration appeared to be insufficient to prevent systemic endothelial injury, which may in part account for the refractory response of nerve function to aminoguanidine treatment.

Aminoguanidine is a potent inhibitor of AGE formation [1]. A number of laboratories have reported that in diabetic subjects peripheral nerve proteins including myelin [16], axonal cytoskeletons [17, 18] and matrix proteins [19] undergo glycation with subsequent AGE formation. Recent ultrastructural studies have shown AGE deposits in both nerve fibres and endoneurial microvasculature in nerves from human diabetic patients [20]. The axonal atrophy and decreased MNCV detected in chronically diabetic rats has been attributed to the long-term accumulation of AGE in the peripheral nerve, based on the observation that treatment with aminoguanidine for 16 weeks prevented these abnormalities [5]. The restoration of MNCV delay in aminoguanidine-treated diabetic rats detected in this study may therefore be ascribed to the protection of nerve structural proteins as well as enzymatic proteins including (Na⁺, K⁺)-ATPase being denatured. It may also be considered that inhibition of AGE accumulation in the vascular wall by aminoguanidine treatment may have improved

endoneurial blood flow which in turn increased MNCV [3, 4]. With a relatively short duration of experimental diabetes, the recovery of nerve conduction deficits by aminoguanidine is well associated with improvement of nerve blood flow [4]. In our study, however, we did not measure the nerve blood flow and therefore it is still unclear whether the endothelial cell injury accompanies altered endoneurial blood flow.

Independent of the potential of aminoguanidine to protect the endothelial injury, there are several other possible mechanisms that could account for how aminoguanidine restored the impairment of MNCV and $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity in diabetic rats. Since aminoguanidine does not affect the polyol pathway [4], metabolic cascades of *myo*-inositol depletion, altered activity of diacylglycerol and protein kinase C are difficult to explain for the improved $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity [21]. An alternate metabolic pathway from phospholipase A_2 to arachidonic acid by activation of protein kinase C could be involved because $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity may in part be suppressed by this pathway under hyperglycaemia [22]. It can also be speculated that association of nitric oxide (NO) with cyclic AMP (cAMP) in both neural and vascular tissues may have an important role in the restoration of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity in the aminoguanidine-treated group. In vitro experiments on neuroblastoma cells have shown recently [23] that stimulation of cAMP with subsequent activation of protein kinase A promotes NO production, which in turn enhances $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity. Because AGE decreases tissue NO and cAMP concentrations [24, 25], inhibition of AGE formation by treatment with aminoguanidine may preserve $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity directly in neuronal tissues or indirectly by correction of nerve blood flow. This notion is supported by recent studies that show that treatment with aminoguanidine together with NO-inhibitor diminished the ameliorative effects of aminoguanidine to improve MNCV delay in diabetic rats [26].

It is not clear to what extent alterations of endoneurial vessels in the peripheral nerve contribute to the increased serum thrombomodulin concentrations in diabetic rats. Such concentrations derived from systemic vessels may not be a sensitive measure of the extent of endothelial damage in the peripheral nerve. Our immunohistochemical studies did not identify any differences in the expression of thrombomodulin antigens in the peripheral nerve with diabetes nor with aminoguanidine or insulin treatment. More sensitive methods or more specific markers are perhaps required to evaluate the abnormalities occurring in the endothelial cells of endoneurial microvessels in diabetic rats. Nevertheless, the presence of thrombomodulin on the endothelial cells in the peripheral nerve and increased serum thrombomodulin

concentrations in diabetic rats do not preclude the possibility that endoneurial microvessels undergo endothelial injury, influencing the development of neuropathy. Our observations – that insulin treatment corrected the increase of serum thrombomodulin concentrations and more effectively preserved nerve function – supports the concept that endothelial injury does contribute to nerve dysfunction in diabetic neuropathy. With the experimental protocol used in this experiment, we cannot eliminate the possibility that treatment with aminoguanidine for a longer duration or at higher doses may protect endothelial cells and prevent an increase in serum thrombomodulin concentrations.

Under ambient hyperglycaemia, oxidative stress causes endothelial dysfunction and cell injury [27]. Lack of the effects of aminoguanidine on the endothelial injury in our study appears to be in keeping with results showing an improvement of nerve blood flow with a restoration of the decreased MNCV in aminoguanidine-treated diabetic rats [3]. Increased levels of oxygen free radical activity as measured by conjugated dienes and hydroperoxides in nerve tissues in diabetic rats were also reported but no statistically significant changes of these levels with aminoguanidine treatment could be found [3]. The latter observations suggest that glycation-unrelated oxidative stress by glucose oxidation as well as excessive flux of polyol pathway could be responsible for the sustained production of free radicals [21, 28–30]. It is therefore likely that aminoguanidine alone cannot prevent completely the development of diabetic neuropathy and other treatment modalities like anti-oxidants as well as aldose reductase inhibitors may be required when a complete return to normal blood glucose cannot be attained.

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