Functional and structural abnormalities in the nerves of Type I diabetic baboons: aminoguanidine treatment does not improve nerve function

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Abstract

Aims/hypothesis. To improve understanding of the pathophysiology of diabetic neuropathy and to establish a primate model for experimental studies, we examined nerve changes in baboons with Type I (insulin-dependent) diabetes mellitus. We also examined the effect of aminoguanidine (an inhibitor of the formation of advanced glycation end products) on nerve function.

Methods. Male baboons (*Papio hamadryas*) were assigned to four groups; control, diabetic, control and diabetic treated with aminoguanidine. Diabetes was induced with streptozotocin (60 mg/kg, intravenous). Insulin and aminoguanidine (10 mg/kg) were injected subcutaneously daily. Motor and sensory nerve conduction velocity was measured using standard techniques. Autonomic function was examined by measuring heart rate response to positional change. Sural nerve morphometry was analysed in the diabetic group (mean duration 5.5 years) along with their age-matched controls.

Results. The diabetic groups were smaller in size with a mean HbA_{1c} of $8.9 \pm 1.2\%$. The nerve conduction

velocity and heart rate response was reduced in the diabetic groups. Morphometric analysis of the diabetic sural nerve showed smaller axon diameter ($2.99 \pm 0.06 \mu\text{m}$ vs $3.29 \pm 0.06 \mu\text{m}$; $p < 0.01$) accompanied by thinner myelin ($1.02 \pm 0.02 \mu\text{m}$ vs $1.15 \pm 0.02 \mu\text{m}$, $p < 0.01$) with no change in the axon density. Treatment with aminoguanidine for 3 years had no effect on glycaemic control and did not restore conduction velocity or autonomic dysfunction in the diabetic animals, contrary to the studies in rats. **Conclusions/interpretation.** These results show that the primate is a good model to study diabetic neuropathy and suggest that the accumulation of advanced glycation end products are not an early mechanism of nerve damage in this disorder. [Diabetologia (2000) 43: 110–116]

Keywords Diabetic complications, neuropathy, autonomic, nerve conduction velocity, aminoguanidine, monkey, morphometry, myelin, advanced glycation end products.

Diabetic neuropathy is a serious complication affecting as many as 60% of diabetic patients [1]. Its clinical

manifestations can vary from reduced nerve conduction velocity and sensory deficits to autonomic disturbances associated with increased mortality [2, 3]. Despite numerous research efforts, the pathogenesis of diabetic neuropathy remains incompletely understood. Proposed mechanisms include microvascular abnormalities, altered Schwann cell or neuronal metabolism, deficiencies of neurotrophic substances and slowing of axonal transport [4–7]. In diabetic patients, changes in function of peripheral and autonomic nerves can be detected very early in the dis-

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Abbreviations: MNCV, Motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; AGE, advanced glycation end products; STZ, streptozotocin.

ease. There is a characteristic slowing of both motor and sensory nerve conduction velocity which improves with better metabolic control [6, 8]. Abnormalities of heart rate variation have also been reported [2, 9]. These functional abnormalities precede overt symptoms by many years. In the more advanced phase of diabetes, sensory symptoms and autonomic disturbance often become clinical problems. At this stage, nerve conduction and heart rate variation are usually abnormal and accompanied by pathological changes of demyelination, decrease in axon diameter, fibre loss and nodal swelling [10–12].

Much less is known about the very early structural changes in patients with diabetes as biopsy specimens are rarely available [13]. In the rat, several studies have reported electrophysiological and structural changes occurring within months of induction of diabetes but the differences in anatomy and physiology of rats from humans make this model not entirely satisfactory for studying the human disease [5, 14]. Therefore we examined nerve changes in the first 5 years of diabetes in our established colony of baboons with Type I (insulin-dependent) diabetes mellitus.

In addition, a group of diabetic and control animals treated with aminoguanidine were studied. It has been proposed that the excess formation of advanced glycation end products (AGEs) in diabetes causes cellular dysfunction and damage, leading to diabetic complications [15, 16]. Aminoguanidine has been shown to inhibit the formation of AGEs in tissues including the nerve [17, 18]. A number of studies have reported that treatment with aminoguanidine in diabetic rats partially restored nerve conduction velocity and was associated with preservation of axonal size [18–22]. Our aim was to examine the effects of aminoguanidine on the functional nerve changes in the baboon in the early stages of diabetes.

Materials and methods

Animals. Male baboons (*Papio hamadryas*) were used in a long-term diabetic study and the husbandry of these animals has been reported previously [23]. In brief, the animals were housed in an outdoor enclosure at the National Baboon Colony in accordance with the guidelines of the Institutional Animal Welfare Committee, Animal Research Review Panel and the National Health and Medical Research Council [24, 25]. The baboons were fed twice daily at times that corresponded to peak insulin concentrations. The diet consisted of a ration of bread, fruit and vegetables, primate pellets (Doust and Rabidge, NSW, Australia), nuts and seeds. They had free access to water. Baboons were assigned randomly at 2.5 years of age (5 kg) to controls ($n = 8$), diabetic ($n = 10$), diabetics treated with aminoguanidine ($n = 5$) and controls treated with aminoguanidine ($n = 5$). Due to a limitation of the numbers of animals of the same age, it was necessary for an aminoguanidine-treated control group to be started at 3 years of age (7.5 kg). Diabetes was induced by an intravenous injection of freshly

prepared streptozotocin (STZ, Sigma, NSW, Australia) (60 mg/kg, in 0.1 mol/l citrate buffer, pH 4.5). After several days of consistently high fasting plasma glucose concentrations (> 25 mmol/l), the animals were started on a once (given between 0800–0900 hours) daily injection of long and short acting insulin with dosage titrated to maintain moderate hyperglycaemia. Each month the animals were weighed and had blood taken for biochemistry and haematology. Plasma glucose was measured by glucose oxidase and glycated haemoglobin was measured by high performance liquid chromatography (Bio-Rad, Richmond, Calif., USA). All procedures were carried out under ketamine anaesthesia (Ketalar, Parke Davis, NSW, Australia) either intramuscularly (6 mg/kg) for short procedures or intravenously ($13 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) for longer ones.

The aminoguanidine treated groups received daily (between 0800 and 0900 hours) subcutaneous injections of aminoguanidine at 10 mg/kg (supplied by Alteon, N.J., USA) from the time they entered the study. The concentration of aminoguanidine in the blood of control and diabetic animals was measured as described previously [26]. Otherwise the aminoguanidine-treated animals were handled identically to the untreated groups.

Nerve conduction velocity. Motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) were measured in the sciatic and sural nerves (respectively) of each experimental animal, using methods modelled on those described for humans [27]. The skin temperature of the animals was monitored by a thermistor (Digi-Sense, Model 8528–40, Cole-Parmer Instrument Co, NSW, Australia), placed on the sole of the foot and maintained at or above 36°C . The nerves were stimulated with 27 gauge needle electrodes, using square wave impulses of 0.5 ms duration generated at 2 pulses per second by the MS91 (Medelec, Surrey, UK). For MNCV, compound muscle action potentials were measured from the dorsal surface of the foot after stimulation from the sciatic notch and the popliteal fossa. The interelectrode distance was measured to the nearest millimeter and used to eliminate variability from the neuromuscular junction. The SNCV was measured orthodromically in each sural nerve. Thirty two traces were recorded and averaged by the MacLab (Analog Digital Instruments Pty., Coopertino, Calif., USA) to eliminate noise. All results were stored and analysed on the MacLab. The MNCV and SNCV were calculated for both legs and the mean used as the conduction velocity.

To define more precisely the effects of age on nerve conduction, 19 male animals (aged 1 year to 8 years) from the colony had MNCV and SNCV measurement. As there is no published data on conduction velocity in different ages of baboons, it was necessary to establish our own values over that period.

Autonomic function. Autonomic function was assessed by measuring the heart rate response to postural change when the mean duration of diabetes was 3.9 ± 1.7 years. A regimen of bolus intramuscular ketamine injection (6 mg/kg) followed by low dose intravenous infusion ($0.13 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$), was used to stabilise the heart rate before beginning the study as described previously [28]. Ketamine was selected as the anaesthetic agent to minimise the effects on the cardiovascular system [28, 29]. After the initial sedation, there was a 15-min period to stabilise the heart rate. This was followed by 5 min of recording before and after the animal was positioned upright on a tilt table specially designed for similar work in humans. The heart rate variation was calculated as the percentage change in beats/min in the erect and supine position.

Table 1. Clinical variables of the baboons

	Duration of diabetes (years)	Fasting plasma glucose (mmol/L)	HbA _{1c} (%)
Control (<i>n</i> = 8)	–	4.8 ± 0.4	4.6 ± 0.4
Diabetic (<i>n</i> = 10)	4.8 ± 1.7	21.6 ± 5.1 ^a	8.9 ± 1.2 ^a
Control + amino- guanidine (<i>n</i> = 5)	–	4.9 ± 0.3	4.6 ± 0.1
Diabetic + amino- guanidine (<i>n</i> = 5)	2.9 ± 0.1	23.4 ± 2.4 ^a	8.5 ± 0.9 ^a

Values are in means ± SD. ^a *P* < 0.001 compared with respective control animals

Nerve morphometric analysis. A biopsy specimen was taken from the right sural nerve and processed for transmission electromicroscopy (TEM). The nerve section was fixed in 2% acrolein and 2% glutaraldehyde in 0.1 mol/l phosphate buffer at pH 7.3 and post fixed in 1% osmium tetroxide in 0.1 mol/l phosphate buffer. Specimens were dehydrated, infiltrated and embedded in Spurr's resin. Thin sections were cut on an Ultracut (Leica, Vienna, Austria) and mounted onto 400 mesh copper grids, stained with uranyl acetate solution and lead citrate. Sections were viewed on a CM12 (Philips, Eindhoven, The Netherlands) and digital electron micrographs (× 4400) were recorded on a Macintosh computer with a Digital Micrograph program (Gatan, Pleasanton, Calif., USA). We studied seven diabetic animals with a mean diabetic duration of 5.2 ± 0.8 years and seven age-matched controls. Each biopsy specimen usually contained three to five fascicles and all the fibres not obscured by grid bars were measured. Myelinated axons were measured for axon diameter and myelin thickness using the PrismView program (Analytical Vision, Raleigh, N.C., USA) on a Macintosh computer. Axon counts were estimated by counting all profiles not obscured by the grid and calculated as number of axons per mm² and corrected for edge effect [31]. The g ratio of the fibres, which is myelin sheath thickness divided by the axon diameter, was calculated for each myelinated axon. The observer was blinded to the diabetic status of the specimens.

Statistical analysis. All data are presented as mean ± standard deviation (SD). Statistical significance between groups was analysed using ANOVA. A *p* value of less than 0.01 was considered significant.

Results

Biochemical and clinical profile. The STZ-induced diabetic animals showed consistent fasting hyperglycaemia throughout the study (Table 1). Insulin treatment maintained a mean HbA_{1c} in the diabetic groups similar to that seen in humans with Type I diabetes. The baseline weight at the beginning of the study was similar in all groups. The diabetic groups had, however, a slower growth rate and at 6 years were considerably less in weight (Fig. 1).

Nerve conduction studies. The electrophysiological results are shown in Figures 2 and 3. In the controls,

Fig. 1. The weights of the control animals (○), controls treated with aminoguanidine (●), diabetics (△) and diabetics treated with aminoguanidine (▲) from 2.5 years of age (start of the study) to 6 years (4.5 years duration of diabetes). Error bars are SD. **p* < 0.001 from the controls

Fig. 2. The change of MNCV with age (years) in the controls (○), controls treated with aminoguanidine (●), diabetics (△) and diabetics treated with aminoguanidine (▲). Error bars are SD. **p* < 0.01 from the controls

MNCV and SNCV showed a steep increase from 1 to 3 years of age, thereafter the curve plateaus. The diabetic group had a significantly lower MNCV and SNCV (*p* < 0.01). The amplitude of the sensory evoked potential was not significantly different between the control and diabetic animals (0.25 ± 0.08 and 0.27 ± 0.07 μV, respectively).

Fig. 3. The change of SNCV with age (years) in the controls (○), controls treated with aminoguanidine (●), diabetics (△) and diabetics treated with aminoguanidine (▲). Error bars are SD. * $p < 0.01$ from the controls

Fig. 4. Heart rate regulation in response to change in position from supine to upright in the four groups. The response is in percentage change of heart rate from the supine position. * $p < 0.01$ from the controls

Autonomic function. The autonomic function test showed that the diabetic group had an attenuated increase in heart rate response when moved upright from the supine position (Fig. 4).

Morphometric analyses. The diabetic group had a mean myelinated axon diameter ($2.99 \pm 0.06 \mu\text{m}$) which was significantly lower than the control group

Fig. 5. Frequency histogram of the myelinated axon diameter in control and diabetic groups. ■ represents the percentage of myelinated axons at each axon diameter for the control animals. □ represents the values of the diabetic animals

Fig. 6. Frequency histogram of the axon myelin thickness in control and diabetic groups. ■ represents the percentage of myelinated axons at each myelin width for the control animals. □ represents the values of the diabetic animals

($3.29 \pm 0.06 \mu\text{m}$; $p < 0.01$). In the diabetic animals 24% of axons were less than $1.8 \mu\text{m}$, whereas only 17% axons of control groups fell below this diameter. This is accompanied by a reduction of larger fibres (13% between $4.4 \mu\text{m}$ and $6.8 \mu\text{m}$ for the diabetics compared with 20% for the controls) (Fig. 5). The distribution of myelin thickness of the diabetic nerve fibres showed a similar shift, the mean myelin thickness was significantly lower in the diabetic animals than controls ($1.02 \pm 0.02 \mu\text{m}$ vs $1.15 \pm 0.02 \mu\text{m}$, $p < 0.01$) (Fig. 6). There was no significant difference between the g ratios of the diabetic (0.45 ± 0.1) and control (0.39 ± 0.1) groups nor any significant difference in nerve fibre density for the diabetic ($0.48 \pm 0.22 \times 10^3 \text{ mm}^{-2}$) and the controls ($0.51 \pm 0.21 \times 10^3 \text{ mm}^{-2}$) groups.

Fig. 7. Aminoguanidine concentration in the plasma of control and diabetic baboons after a subcutaneous injection of aminoguanidine (10 mg/kg, $n = 9$)

Aminoguanidine treatment. Pharmacokinetic study showed that aminoguanidine injected according to our protocol increased plasma aminoguanidine concentration within 30 min which remained detectable at 24 h (1.4 ± 1.06 µg/ml) (Fig. 7). Treatment had no effect on glycaemic control (Table 1). Although the aminoguanidine-treated controls and diabetic groups were slightly lighter than their respective untreated groups, the difference never reached statistical significance (Fig. 1). The MNCV and SNCV of the diabetic animals were not restored by 3 years of aminoguanidine treatment and there was no statistically significant effects on the control MNCV and SNCV treated with aminoguanidine (Fig. 2, 3). Aminoguanidine treatment had no effect on amplitude of the sensory action potential in the diabetic and control groups that were treated (0.25 ± 0.06 and 0.26 ± 0.1 µV, respectively) and did not alter the autonomic heart rate response to postural change (Fig. 7).

Discussion

Due to the difficulties of obtaining tissue samples in humans, there is still an incomplete knowledge of the pathogenesis and early changes in diabetic neuropathy. Primates have been used previously to examine complications of diabetes; their phylogenetic closeness to humans makes it easier to draw analogies between the species [23, 32–34]. The longevity of the primate permits long-term serial studies which are not possible in the rodent, the traditional experimental model of diabetes. This led us to document in a baboon model of Type I diabetes the functional and structural changes of the peripheral nerve in the first

few years after induction of diabetes. Our results showed that there is a definite reduction in MNCV and SNCV by 3 years of diabetes with demonstrable morphometric changes at 5 years. Similar changes of nerve conduction have been reported in rats but as diabetes is usually induced in a phase of rapid body growth, it is difficult to distinguish the effects of diabetes from that of retarded maturation in this model [5, 14]. This problem has been minimized in our study; the longevity of baboons allowed the induction of diabetes to be at an age when the increase in nerve conduction had plateaued, as shown by the measurements on the controls (Fig. 2, 3).

Another advantage of the primate model is the availability of samples for examination of nerve fibre structure in the first few years of diabetes. In the sciatic nerves of diabetic rats, there are obvious morphological changes within 5 months of induction [18, 35]. Interpretation of these results needs, however, to take into account that these rats are often extremely hyperglycaemic and in the rapid stage of nerve growth [14, 36]. In humans, sural nerve biopsy specimens taken in the later stages of diabetes, usually when neuropathy is a clinical problem, have provided information on structural abnormalities. The loss of large axons, a shift towards smaller myelinated fibres and demyelination are characteristic findings [10, 12, 37]. By comparison, there is little information on the changes in the first few years. The results of sural nerve biopsies from eight children with subtle neurophysiological changes (a reduction in NCV and amplitude), when the mean duration of diabetes was 8.5 years have been presented in abstract form [13]. Data from teased nerve fibres showed segmental demyelination and paranodal abnormalities but no axonal degeneration in diabetic subjects. These changes were not accompanied by alterations in myelinated fibre density, myelinated fibre area and axonal area. The duration of diabetes of our diabetic baboons was 5.2 years and the metabolic control was typical of young Type I diabetic patients. Morphometric studies in the sural nerves showed reduced myelin thickness paralleled by a commensurate fall in mean axon diameter. Although there are differences in the findings reported in the abstract and ours, both support the notion that in patients receiving conventional therapy, nerve changes are already evident within a few years of Type I diabetes. Only one other group have studied nerve morphology in a primate model of Type I diabetes [33]. They examined the sural nerve of macaques 12 to 36 months after induction of diabetes with STZ. They found mild changes to both myelinated and non-myelinated axons and concluded that due to the slow onset and mildness of pathology, this primate model was inferior to that of the rat for study of diabetic neuropathy. The findings reported in the abstract and ours indicate, however, that development of neuropathy in humans is indeed

slow. In comparison to the relatively minor changes to morphology, nerve conduction velocities are much more affected. This dichotomy is typical of diabetic neuropathy and clearly indicates other factors affect nerve conduction, probably subtle biochemical or vascular changes or both. Morphometric changes of endoneurial blood vessels are also thought to be important in the pathogenesis of diabetic neuropathy and are currently being studied in these samples. The baboon model in our opinion appears to be appropriate for monitoring the development of neuropathy and the testing of treatment strategies in humans.

Clinically, autonomic neuropathy is a late complication in diabetes associated with high morbidity and mortality. Subtle changes of autonomic function can, however, be shown many years earlier [2]. There are two reports of a reduced heart rate response to postural change and deep breathing in 30% of children with diabetes of less than 10 years duration [9, 38]. Although structural changes of the autonomic nerve have been shown in diabetic rats, there has not been any suitable animal model for studying autonomic dysfunction [21]. The diabetic baboons showed a decrease in heart rate response similar to that seen in humans. Therefore, the baboon model of diabetes is also appropriate for studying the pathogenesis and treatment of autonomic neuropathy.

Advanced glycation end products have been found in the nerves of diabetic rats and have been postulated to have a pathogenetic role in neuropathy. They can disturb cell function in a number of ways, amongst these are effects on cross-linking of molecules, nitric oxide synthesis and action, generation of cytokines and production of free radicals [16]. Aminoguanidine inhibits the formation of AGEs and possibly prevent diabetic complications through one or more of these pathways. The trials of aminoguanidine treatment in humans with late stage diabetic complications are ongoing, although preliminary evidence indicates that it is not effective in advanced renal disease [39]. In our study of long-term aminoguanidine treatment in diabetic primates aiming at the prevention of diabetic complications in the early phase, both groups of baboons treated with aminoguanidine had similar glycaemic control but slightly lower weight gain than their untreated counterparts, a phenomenon also observed in rats [21, 40]. Despite 3 years of treatment from the onset of diabetes, aminoguanidine did not prevent the impairment of MNCV, SNCV or heart rate response of diabetic animals. Its effects on nerve morphometry have not been assessed at this stage because aminoguanidine treated animals have not reached the duration of diabetes for nerve biopsies. Electrophysiological results lead us, however, to believe that aminoguanidine will have little if any effect on structural changes. The inability of aminoguanidine to restore nerve function in baboons differs from some of the studies

in rats that showed treatment to be beneficial although the results are conflicting [18, 19, 22, 41, 42]. One study found that diabetic rats given 50 mg/kg aminoguanidine in the drinking water for 16 weeks had NCV restored although doses of 10 and 25 mg/kg were ineffective [19]. Another that diabetic rats injected intraperitoneally with aminoguanidine ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) had NCV restored but not when aminoguanidine (1 g/l) was given in drinking water [22]. Yet another reported, however, that aminoguanidine (1 g/l) in the drinking water did restore NCV in diabetic animals [40]. Apart from inhibiting AGEs formation, aminoguanidine has other actions which could be different in the rodents and primates. Due to the anatomy of rats, measurement of NCV is more difficult than in larger animals. These are all factors that may contribute to the differing results. The dosage of aminoguanidine used for our study was shown by pharmacokinetic studies to attain a plasma concentration clearly detectable after 24 h and similar to that reached in humans and rats at an equivalent dose [43, 44]. To be completely certain that aminoguanidine treatment in our hands has been effective in preventing formation of AGEs, it is necessary to measure these products in peripheral nerves. This is difficult due to the limited size of tissue samples but we are currently measuring the level of AGEs in renal biopsy samples of these baboons.

There has been much research on functional nerve changes in the early stages of diabetic neuropathy. There is, however, little information on early structural changes that could be the key to understand the long-term damage and the development of clinical disease. In this study we were able to examine the function of the peripheral and autonomic nerve in light of the morphometric changes of the sural nerve in the first 5 years of diabetes. The inability of aminoguanidine to prevent the decrease in nerve conduction velocity in the primate suggests that formation of AGEs is not the major cause of functional nerve changes in the early stages of diabetes.

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