Overt Diabetic Neuropathy: Repair of Axo-glial Dysjunction and Axonal Atrophy by Aldose Reductase Inhibition and its Correlation to Improvement in Nerve Conduction Velocity

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Clinically overt diabetic neuropathy is characterized by neuroanatomical changes of the node of Ranvier and myelinated axons, and by decreased nerve conduction velocity. Sural nerve biopsies were obtained from 16 neuropathic diabetic patients participating in a 12-month randomized, placebo-controlled, double-blind clinical trial of the aldose reductase inhibitor sorbinil. One sural nerve biopsy was obtained at baseline and a second biopsy at the termination of the trial. Ten sorbinil-treated patients showed significant improvement in axo-glial dysjunction, a characteristic lesion of the node of Ranvier. Axonal atrophy assessed by three independent morphometric techniques also exhibited significant recovery in the sorbinil-treated patients. No change was demonstrated in any of these structural parameters in six placebo-treated patients. The improvement in sural nerve conduction velocity in sorbinil-treated patients correlated with the product of the quantitative improvements in axo-glial dysjunction and axonal atrophy. We conclude that the activated polyol-pathway plays a sustaining role in nerve fibre damage in diabetic neuropathy, and that structural lesions such as axo-glial dysjunction and axonal atrophy which are reversible following intervention with an aldose reductase inhibitor, constitute the morphological basis for nerve conduction slowing in overt diabetic neuropathy.

KEY WORDS Diabetic neuropathy Axo-glial dysjunction Axonal atrophy
Sural nerve conduction velocity Aldose reductase inhibition

Introduction

The exact pathogenesis of diabetic neuropathy remains controversial, although metabolic abnormalities related to hyperglycaemia, microangiopathic changes, and undefined independent genetic and environmental factors are thought to be contributory.[1-6] Several epidemiological studies associate the incidence of clinical neuropathy with duration of diabetes and poor glycaemic control, suggesting cumulative hyperglycaemia as a major contributing factor.[7-9]

Data from animal and human studies have implicated activation of the polyol-pathway by hyperglycaemia as probably the most important initiating abnormality responsible for diabetic neuropathy.[10-21] In animal studies relationships have been demonstrated between polyol-pathway related metabolic abnormalities and slowed nerve conduction velocity in acutely diabetic rats, [3,10,] [12,13,19] and between structural lesions and slowed nerve conduction velocity in chronically diabetic animals,[22,][23] suggesting an acute metabolic and a chronic structural genesis for the impaired nerve function in diabetic neuropathy.[10]

We have recently demonstrated that the characteristic nodal abnormality axo-glial dysjunction correlates closely with slowed peripheral nerve conduction velocity, [22] and that axo-glial dysjunction and axonal atrophy of the optic nerve are major structural determinants for prolonged latencies of the visual evoked potential in chronically diabetic BB/W-rats.[23]

In the present study, similar relationships were sought between structural lesions and impaired nerve function in diabetic patients. We employed in part previously published data on sural nerve morphometry and nerve conduction velocity obtained from patients participating in a double-blind placebo-controlled clinical trial of the aldose reductase inhibitor (ARI) sorbinil.[21,24]

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Patients and Methods

Patients

The entrance criteria, patient population, and study design for the 12-month clinical trial of the drug sorbinil in patients with clinical diabetic distal symmetrical polyneuropathy have previously been described in detail.[21, 24] In brief, patients were 18 to 65 years of age (mean 47.1 ± 3.6 SEM years). They had had diabetes as defined by the National Diabetes Data Group [25] for 1 to 20 years (mean 10.0 ± 1.3 years), and clinically evident neuropathy for at least 6 months (mean 2.8 ± 0.8 years) accompanied by an elevated haemoglobin A_1c level (mean 8.0 ± 0.4%).[24]

After having consented to participate in the study and undergone a 2-month single-blind placebo 'run-in' period during which baseline neurological function was assessed, patients were randomly assigned to either continued placebo or active treatment with sorbinil (250 mg day^{-1}) in a 1:2 ratio. Neurological assessments included symptom questionnaires, scored clinical neurological examination, automated tactile and thermal perception threshold levels, and standardized electrophysiological testing including sural sensory nerve conduction velocity and compound action potential amplitude. Of 31 patients entering into the study, 27 completed the trial and 16 patients consented to both a baseline and a follow-up sural nerve biopsy at the termination of the trial. The data presented in this communication were derived from this sub-group of 16 re-biopsied patients, whose baseline clinical, demographic, and morphometric characteristics were indistinguishable from the larger group of 27 patients who consented to the baseline biopsy.[21,24] Patients were assessed as having either Type 1 or Type 2 diabetes melliltus according to the National Diabetes Data Group. [25]

Electrophysiological Studies

Sural nerve conduction studies were repeated four times during the run-in period and the mean values were used as baseline values. Sural nerve conduction studies were repeated prior to the second biopsy. Antidromic sural nerve sensory conduction velocity and compound action potential amplitude were measured using surface electrodes placed on the patient's dominant side, opposite to the side of biopsy. Recordings were made in a temperature controlled room at 34°C. A stimulus of 0.2 ms duration and supramaximal (120 %) intensity was applied and the resulting compound action potential was obtained using averaging technique.

Surgical Procedures and Control Biopsies

Fascicular sural nerve biopsies 5 to 6 cm long were obtained under local anaesthesia from the area posterior and proximal to the lateral malleolus of the non-dominant side, not to interfere with serial electrophysiological examinations obtained from the contralateral side.[30] The repeat biopsy at the termination of the trial was obtained through a proximal extension of the original incision, yielding a paired ipsilateral fascicular biopsy 5 to 6 cm long. [35] Control sural nerve biopsies were obtained from 19 carefully age-matched cadaveric organ donors, or at autopsy within 6 h of death. These specimens were used to establish morphometric and teased fibre norms.[24]

Tissue Procurement

Each fascicular sural nerve biopsy was immediately divided into four equally long portions. The two proximal tissue samples were fixed in cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde and postfixed in cacodylate-buffered (pH 7.4) 1% osmium tetroxide. Both specimens were dehydrated in graded concentrations of alcohol; one was embedded in Epon and cross and longitudinal section were used for morphometric analysis, and the other was used for teased fibre preparations in Epon.[24]

Morphometric Assessment of Axo-glial Dysjunction

Nerve biopsies were examined morphometrically for various elements of a sequence of structural changes affecting the node of Ranvier initially identified in the BB-rat,[15] and subsequently demonstrated in human diabetic neuropathy.[24] The most important of these consist of axo-glial dysjunction, characterized by detachment of terminal myelin loops from the axolemma in the paranodal region.[14, 15] The frequency of axo-glial dysjunction was determined from serial longitudinal electron-microscopic sections and defined as the percentage of paranodal terminal myelin loops devoid of junctional complexes, as previously described in detail.[15, 25]

Morphometric Assessment of Axonal Atrophy

Axonal atrophy was assessed by three independent morphometric techniques. Firstly, axon-myelin ratio was assessed from electronmicrographs of 58 ± 3 randomly selected myelinated fibres in cross-sectioned nerve fascicles at a total magnification of 27420 times. The relationship between the natural logarithm of the axonal cross-sectional area and the myelin sheath thickness, expressed as the number of myelin lamellae, was calculated by linear regression analysis. Secondly, axonal atrophy was also assessed by calculating the internodal length–diameter ratio measured from a mean of 63 ± 2 single teased fibres as previously described. [24] The internodal length–diameter ratio increases with axonal shrinkage and decreases with fibre regeneration and remyelination. In this study regenerating and remyelinating fibres were excluded from the internodal length–diameter and the axon–myelin examinations.

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Finally, axonal atrophy was quantified by scoring a mean of 71 ± 6 randomly teased fibres exhibiting excessive myelin wrinkling (thinnest internodal diameter < 50 % of the thickest diameter) as previously described.[24, 25]

Correlations Between Axo-glial Dysjunction Axonal Atrophy and Nerve Conduction Velocity

The correlations between sural nerve conduction velocity and the product of the natural logarithms of axo-glial dysjunction and axonal atrophy as the independent variable were performed using the baseline data and the change between baseline and follow-up values. Axonal atrophy was expressed by either excessive myelin wrinkling, myelin–axon ratio or internodal length–diameter ratio. For these calculations the axon–myelin ratio was inverted in order for it to numerically move in the same direction as excessive myelin wrinkling and internodal length–diameter ratio following treatment.

Statistical Analysis

All data are expressed as means ± SEM. The statistical significance of change from baseline to follow-up in morphometric parameters and sural nerve conduction velocity for each treatment group was assessed by paired two-tailed t-tests. The probabilities that differences between baseline and follow-up values occurred by chance alone in the sorbinil-treated patients is expressed as P_s and in the placebo-treated subjects as P_c. The treatment effect defined as the difference betwen the respective baseline to follow-up differences in the two treatment groups was assessed by the two-tailed Behrens-Fisher test which does not assume equality of variances and reported as P_t. The correlations between nerve conduction velocity and axo-glial dysjunction and axonal atrophy were performed by linear regression analysis by the least squares method. Patients with Type 1 and Type 2 diabetes did not differ at baseline with respect to most morphometric parameters measured.[24] Therefore, follow-up data of patients with Type 1 and Type 2 diabetes were pooled except when parameters differed significantly at baseline in which case the data were presented both as aggregate and separately. Since myelinated fibre regeneration was significantly increased following sorbinil treatment, [21] the frequencies of scored teased fibre abnormalities including excessive myelin wrinkling were calculated as percentages of all fibres minus the number of regenerated or remyelinated fibres, in order to avoid a dilution effect contributed by regenerated fibres.[26]

Results

Effect of ARI Treatment on Nerve Conduction Velocity

Patients randomized to sorbinil and placebo-treatment showed similar sural nerve conduction velocities at baseline.[21] Following 12 months of sorbinil treatment there was a significant ($P_s<0.05$) improvement in sural nerve conduction velocity in patients treated with sorbinil, whereas placebo-treated patients showed a non-significant decrease in nerve conduction, resulting in a significant treatment effect ($P_t<0.05$) (Table 1).

Effect of ARI Treatment on Axo-glial Dysjunction

Patients randomized to sorbinil treatment and those assigned to placebo treatment both showed an increase in the frequency of axo-glial dysjunction at baseline compared with age-matched normal controls (12.3 ± 1.3 %). At the termination of the study the patients receiving sorbinil showed a significant decrease in their frequencies of axo-glial dysjunction ($P_s<0.02$) (Table 1), whereas patients receiving placebo treatment showed no significant change over the 12-month period ($P_c=NS$) (Table 1), resulting in a significant treatment effect ($P_t<0.03$).

As previously noted axo-glial dysjunction appeared to be more prevalent in the neuropathy accompanying Type 1 diabetes than in that associated with Type 2 diabetes.[24] This difference at baseline was also evident in the present subgroup of rebiopsied patients ($p<0.005$). Hence, the effect of sorbinil-treatment on axo-glial dysjunction in Type 1 and Type 2 diabetic patients was considered separately. Type 1 diabetic patients ($n=5$) receiving sorbinil treatment showed an improvement in axo-glial dysjunction from 33.9 ± 4.3 % at baseline to 16.8 ± 2.3 % at follow-up ($p<0.01$), whereas Type 2 diabetic patients ($n=5$) showed only trivial improvements from 15.2 ± 1.0 % to 13.3 ± 1.1 %, respectively ($p=NS$).

Effect of ARI Treatment on Axonal Atrophy

Axonal atrophy as manifested by excessive myelin wrinkling was increased approximately 10-fold in both sorbinil and placebo-treated patients at baseline compared to age-matched controls. The frequency of excessive myelin wrinkling decreased significantly in sorbinil-treated patients (Table 1) ($P_s<0.0001$) with no differential effect in Type 1 and Type 2 diabetic patients. No change was noted in placebo-treated patients ($P_c=NS$) over the 12-month study period (Table 1), rendering a significant treatment effect ($P_t<0.007$).

Axonal atrophy as assessed by regression analysis of the relationship between internodal length and internodal fibre diameter revealed similar patterns of change in sorbinil-and placebo-treated patients at baseline. Twelve months of sorbinil treatment significantly improved ($P_s<0.0001$) the internodal length–diameter ratio, so that it was no longer different from that of age-matched non-diabetic control subjects. No differential effect was obtained in Type 1 and Type 2 diabetic subjects despite a significant difference in internodal length–diameter

Table 1. Changes between baseline and follow-up values of sural nerve conduction velocity, axo-glial dysjunction, and three independent measures of axonal atrophy

| | Sorbinil-treated patients | | Placebo-treated patients | | Treatment effect |
	%	$P_s<$	%	$P_c<$	$P_t<$
Sural nerve conduction velocity (m s^{-1})	2.2 ± 0.2	0.05	−0.8 ± 0.3	NS	0.05
Axo-glial dysjunction (%)	−12.2 ± 3.7	0.02	−1.6 ± 1.0	NS	0.03
Excessive myelin wrinkling (%)	−15.8 ± 1.2	0.0001	−0.7 ± 2.8	NS	0.0007
Internodal length–diameter ratio	26.4 ± 0.7	0.0001	1.0 ± 0.8	NS	0.0002
Axon–myelin ratio	12.5 ± 1.1	0.001	−0.8 ± 0.3	NS	0.05

ratio in the larger baseline material.[24] Placebo-treated patients showed no change in the internodal length–diameter relationship during the study period (P_c=NS) (Table 1). Thus sorbinil treatment appeared to fully correct the internodal length–diameter parameter of axonal atrophy ($P_t<0.0002$).

When axonal atrophy was assessed by axon–myelin ratio, again no significant difference was observed between placebo and sorbinil assigned patients at baseline. Sorbinil treatment for 12 months was associated with a significant ($P_s<0.001$) improvement in the axon–myelin ratio (Table 1) with no differential effect in Type 1 and Type 2 diabetic patients. The axon-myelin ratio remained unchanged in placebo-treated patients (P_c=NS) (Table 1) revealing a significant treatment effect ($P_t<0.05$).

Correlations Between Sural Nerve Conduction Velocity and Axo-glial Dysjunction and Axonal atrophy

The data of all patients with obtainable nerve conduction velocity at baseline (n=23; 9 Type 1 and 14 Type 2 patients) and at follow-up (n=15; 10 treated and 5 placebo patients) were used to calculate the correlations between sural nerve conduction velocity and nodal and axonal structural deficits. Sural nerve conduction velocity showed a significant negative correlation with the product of the natural logarithms of axo-glial dysjunction and axonal atrophy, regardless as to whether the latter was expressed as excessive myelin wrinkling ($p<0.0001$; Figure 1(a), myelin–axon ratio ($p<0.001$; Figure 1(b), or internodal length/diameter ratio ($p<0.001$; Figure 1(c), suggesting that axo-glial dysjunction and axonal atrophy are major determinants for the nerve conduction slowing in clinically overt diabetic neuropathy. This is further supported by the results obtained from the correlations between the change in nerve conduction velocity between baseline and follow-up and the change in the products of the natural logarithms of axo-glial dysjunction and axonal atrophy in the two biopsies. Regardless of how axonal atrophy was assessed, the improvement in sural nerve conduction velocity showed significant correlations with the improvements in axo-glial dysjunction and excessive myelin wrinkling ($p<0.001$; Figure 2(a)), or

myelin–axon ratio ($p<0.001$; Figure 2(b)), or internodal length–diameter ratio ($p<0.001$; Figure 2(c)).

Discussion

Clinically overt diabetic polyneuropathy is characterized by slowing of nerve conduction velocity, decreased action potential amplitude, and a series of partly interrelated neuroanatomical abnormalities. In human diabetic neuropathy as in the neuropathy accompanying diabetes in the rat, the latter appear to involve functionally relevant anatomical structures, such as the nodal apparatus and the axon itself. In this prospective placebo-controlled double-blind clinical trial in patients with clinically overt neuropathy, sorbinil treatment was accompanied by small but statistically significant improvements in sural nerve function as reported previously. [21] In the present communication we have demonstrated that these functional improvements are associated with substantial repair of specific neuroanatomical abnormalities.

Axo-glial dysjunction of the paranodal apparatus correlates in the diabetic rat with the chronic nerve conduction slowing which has been attributed to a decrease in nodal sodium permeability.[14] This in turn probably reflects migration of nodal sodium channels into the internodal area facilitated by the breach in the paranodal junctional barrier through axo-glial dysjunction.[15] Axo-glial dysjunction also constitutes the initiating event in the development of paranodal demyelination, resulting in increased potassium leakage and diminution of the safety factor for the propagation of the nerve impulse, further hampering the nerve conduction velocity.[27] In the present clinical trial sorbinil treatment was accompanied by a significant repair of axo-glial dysjunction, suggesting that shunting of excess glucose through the polyol pathway plays a continuous role in the progression of this structural lesion. This treatment effect was mainly accounted for by the change in the younger Type 1 diabetic patients, who at baseline showed a more severe axo-glial dysjunction compared to the older Type 2 diabetic subjects.[24] The discrepancy in axo-glial dysjunction between Type 1 and Type 2 diabetic subjects has been described earlier and probably reflects a more severe metabolic compromise of periph-
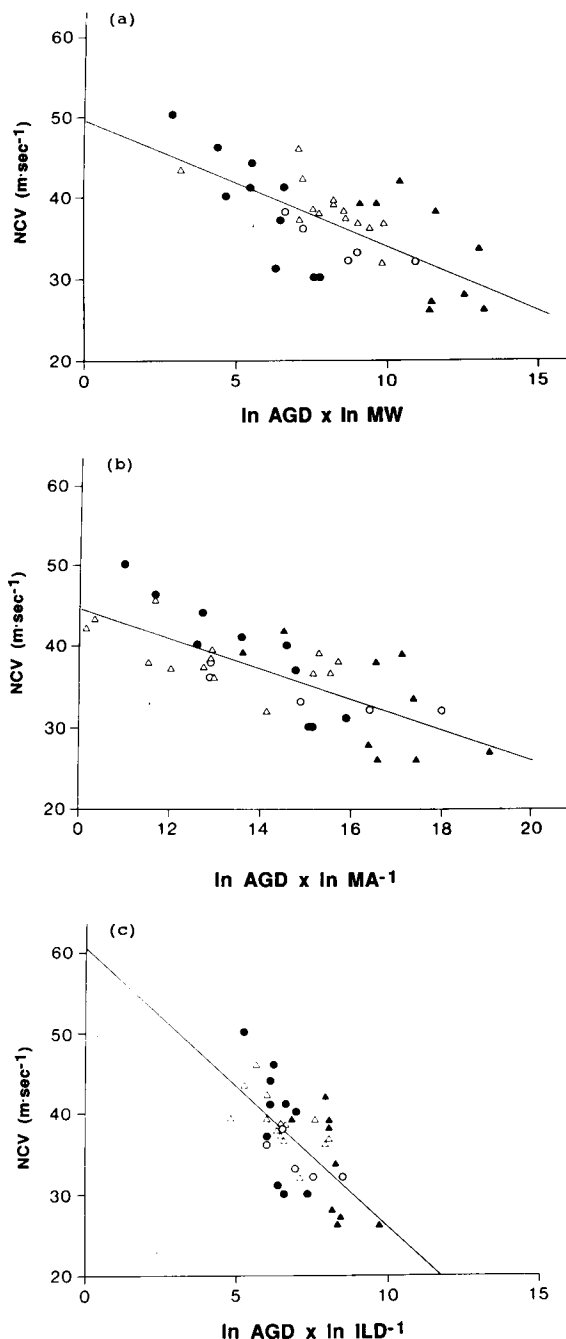
Figure 1. Sural nerve conduction velocity showed significant correlations with the products of the natural logarithms of axo-glial dysjunction (AGD) and axonal atrophy, either this was assessed as (a) excessive myelin wrinkling (MW) ($r^2 = -0.74; p < 0.0001$) or (b) myelin–axon ratio (MA^{-1}) ($r^2 = -0.53; p < 0.001$) or (c) internodal length–diameter ratio (ILD^{-1}) ($r^2 = -0.42; p < 0.001$). ▲ Type 1 diabetic patients ($n=9$); △ type 2 diabetic patients ($n = 14$); ● treated patients ($n = 10$) ○ placebo patients ($n = 5$)

Figure 2. The change in sural nerve conduction velocity ($\triangle$NCV) from baseline to follow-up correlated with the products of the natural logarithms of the change in axo-glial dysjunction ($\triangle$AGD) and axonal atrophy either this was assessed as change in (a) excessive myelin wrinkling ($\triangle$MW) $r^2=0.74$; $p<0.0001$) or (b) myelin–axon ratio ($\triangle$MA^{-1}) ($r^2 = 0.59$; $p < 0.001$) or (c) internodal length–diameter ratio ($\triangle$ ILD^{-1}) ($r^2=0.68$; $p<0.0001$); ● treated patients ($n=10$); ○ placebo patients ($n=5$)

eral nerve in Type 1 diabetic patients.[24] In chronically diabetic BB-rats the severity of axo-glial dysjunction correlates closely with the severity of the chronic nerve conduction defect,[22] suggesting that axo-glial dysjunction is an important determinant for nerve conduction velocity. Such a correlation could not be established in the present human material. Instead the product of axo-glial
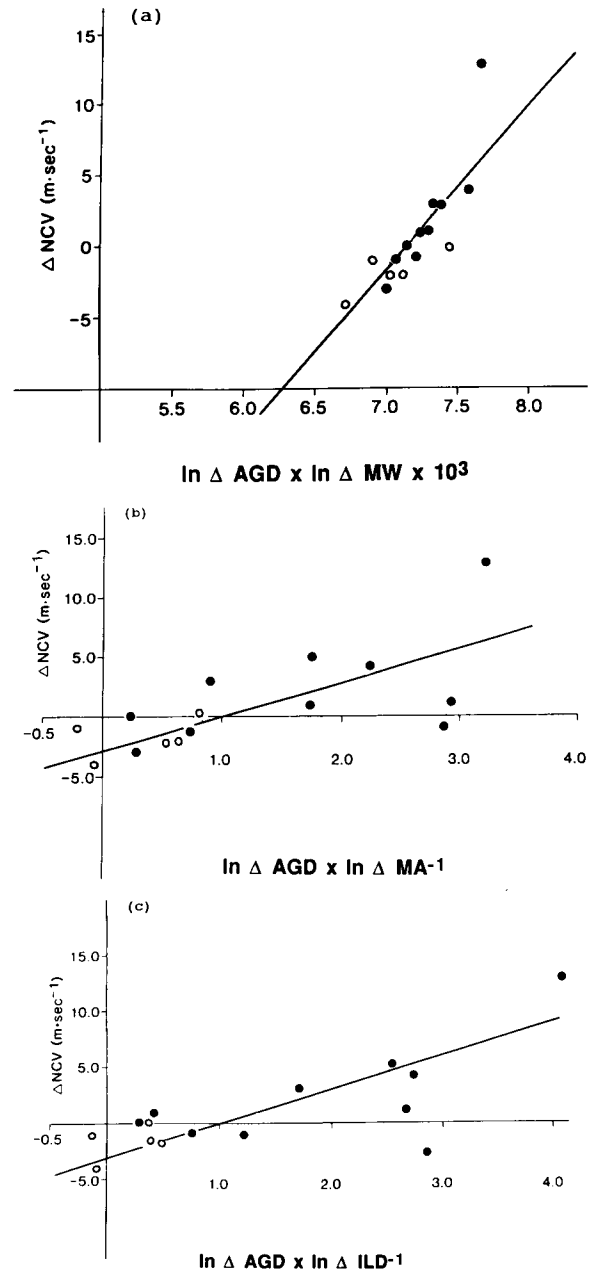
dysjunction and axonal atrophy, assessed by three independent techniques, showed a close negative correlation with nerve conduction velocity, suggesting that besides axo-glial dysjunction, axonal caliber is a major contributor for nerve conduction slowing in clinically overt diabetic neuropathy. This is analogous to the structural–functional relationship in diabetic optic neuropathy in the BB-rat.[23]

Axonal atrophy is a characteristic, though not unique,

abnormality of diabetic symmetric polyneuropathy and has been implicated in the slowing of nerve conduction. [14, 24, 28–31] Twelve months of sorbinil treatment in patients with clinically overt peripheral neuropathy was accompanied by a significant improvement in axonal size. Axonal atrophy in murine diabetic neuropathy is believed to result from slowed axonal transport,[32] as well as impaired protein synthesis, due to diminished sodium-dependent amino acid uptake by the perikaryon consequent to the Na/K-ATPase defect.[33] Since in short-term studies ARI treatment is known to normalize the slowed axonal transport [34] as well as the impaired Na/K-ATPase activity in peripheral nerve,[19, 20] it is not completely unexpected that long-term ARI treatment may improve axonal size as demonstrated in the present study.

In summary, 12 months of ARI therapy in patients with established clinical diabetic polyneuropathy was accompanied by active repair of axo-glial dysjunction and axonal atrophy identified as functionally relevant neuroanatomical lesions, suggesting that hyperglycaemia via its activation of the polyol-pathway plays an active and continuing role in the progressive peripheral nerve damage and closely associated nerve conduction slowing characteristic of diabetic polyneuropathy.

Acknowledgements

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