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Aldose reductase inhibition with imirestat – effects on impulse conduction and insulin-stimulation of Na^+/K^+-adenosine triphosphatase activity in sciatic nerves of streptozotocin-diabetic rats

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Summary. This study describes reduced motor nerve conduction velocity and increased resistance to hypoxia-induced conduction failure in sciatic nerves of rats after four weeks of streptozotocin-induced diabetes (both effects were significant at $p < 0.05$). These changes occurred in the absence of any deficit in the steady-state ouabain-sensitive adenosine triphosphatase (ATPase) activity of sciatic nerve endoneurial homogenates. The addition of 10 nmol/l insulin to endoneurial homogenates from control animals resulted in a 34% increase in ouabain-sensitive ATPase activity and a 19% reduction in ouabain-insensitive ATPase activity (both $p < 0.01$). This stimulation of ouabain-sensitive ATPase activity by insulin did not occur in homogenates from diabetic rats. Treating diabetic rats daily with the aldose reductase inhibitor, imirestat (1 mg/kg) improved nerve conduction velocity ($p < 0.05$) but was without effect upon the resistance to hypoxic conduction blockade or the deficit in insulin-stimulated oubain-sensitive ATPase activity. These data suggest that in streptozotocin-diabetic rats the functional disorders of reduced motor nerve conduction velocity and increased resistance to hypoxic conduction blockade do not share a common aetiology and that impaired nerve conduction is not related to reduced maximal potential oubain-sensitive ATPase activity.

Key words: Diabetes mellitus, diabetic neuropathy, hypoxia, nerve conduction, aldose reductase inhibition, Na^+/K^+-ATPase, insulin.

Reduced motor nerve conduction velocity (MNCV) and increased resistance to ischaemic conduction blockade (RICB) are well established phenomena in diabetic patients [1]. That both may be found in new or recently diagnosed diabetic patients, well before the onset of pathological changes in nerve fibres [2, 3], suggests an underlying metabolic aetiology. This is further supported by reports that both reduced MNCV and increased RICB may be attenuated by instigating more rigorous glycaemic control [2, 4]. Experiments using diabetic animals have therefore been employed to investigate the aetiology of these functional disorders.

Rats exposed to experimental diabetes of a few weeks duration also exhibit reduced MNCV and abnormal RICB [5, 6]. Studies have demonstrated that exaggerated flux of glucose through the sorbitol pathway, underlies the MNCV disorder, perhaps via nerve myo-inositol depletion [5, 7]. The biochemical basis for abnormal RICB in diabetic rats is unclear. Some reports have indicated that prolonged RICB is a consequence of increased availability of energy sources for anaerobic glycolysis in diabetic nerve [8–10]. Others have proposed that the reduced energy requirement of nerve from diabetic animals [11, 12], together with an adaptive enhancement in anaerobic glycolytic capacity resulting from incipient and progressive neuronal hypoxia in diabetes [13], also contribute to the development of RICB. Furthermore, the demonstration of increased RICB in galactose-fed rats [14] and the attenuation of prolonged maintenance of the muscle compound action potential (CAP) amplitude during anoxia in diabetic rats by treatment with an aldose reductase inhibitor [15] suggest that exaggerated flux through the polyol pathway may also be involved in the RICB phenomenon.

The response of rat sciatic nerve to hypoxia has previously been examined by measuring the decline in CAP of stimulated nerves in vitro [6]. This study demonstrated that nerves from alloxan-diabetic rats maintained CAP amplitude for longer durations of hypoxia than control nerves. In the present study we have investigated whether treating diabetic rats with an aldose reductase inhibitor had an effect upon this resistance to hypoxic conduction failure. MNCV was recorded as this represents a polyol-pathway related disorder in diabetic rats, and Na^+/K^+-ATPase activity of nerve homogenates was also measured in recognition of the putative links between polyol pathway flux, Na^+/K^+-ATPase activity and nerve conduction [16]. Furthermore, as recent studies have reported that specific sub-fractions of Na^+/K^+-ATPase activity may be activated by insulin [17] we determined homogenate Na^+/K^+-ATPase activity in both the presence and absence of insulin.

Materials and methods

Experimental organisation

Male Wistar rats (310–360 g) were assigned at random to one of three groups. Animals from two of these groups were then fasted overnight before being made diabetic by an injection of streptozotocin (50 mg/kg i. p.) freshly dissolved in sterile 0.9% NaCl. Three days later, the glucose concentration of blood samples obtained by tail prick, was measured using a strip-operated reflectance meter ('Reflotest-glucose', Boehringer Corporation, London, UK). Streptozotocin-injected rats with a blood glucose concentration of less than 15 mmol/l were excluded from further experimentation. After the confirmation of diabetes, animals from one group of diabetic rats were treated with the aldose reductase inhibitor, imirestat (HOE 843; 2,7-difluorospiro[fluorene-9,4'-imidazolidine]-2',5'-dione; Hoechst Aktiengesellschaft, Pharmaceutical Division, Frankfurt, FRG) at a daily dose of 1.0 mg/kg by gavage for the duration of the study. All animals were housed identically with free access to food and water.

Motor nerve conduction velocity and plasma glucose

Four weeks after the injection of streptozotocin, rats were anaesthetized with halothane (4% in O_2 for induction, 2–3% for maintenance) and a fine thermocouple probe placed adjacent to the mid-femoral region of the left sciatic nerve via a 0.5 mm incision in the flank. The incision was closed and the thermocouple held in place using artery clamps. Nerve temperature was monitored using a digital electronic thermometer (Comark Electronics, Rustington, Sussex, UK) and maintained at 37°C by an infra-red lamp before measurement of distal motor latency in the sciatic nerve: interosseous muscle system as described elsewhere [7]. The thermocouple was then removed, the wound closed with Michel clips and the animal allowed to recover. Two days after this procedure, rats were killed by a blow to the head and bled from the throat to provide a blood sample for the subsequent determination of plasma glucose by spectrophotometric assay (GOD-PERID assay test, Boehringer, Mannheim, FRG). The left sciatic nerve was rapidly exposed from the sciatic notch to the Achilles tendon and its length measured under moderate tension to enable calculation of motor nerve conduction velocity (MNCV).

Nerve function studies in vitro

Both sciatic nerves were removed rapidly, then ligated at the knee joint and proximally at the bifurcation which forms the common spinal roots at L_4 and L_5. The portion between the ligatures (about 4 cm) was incubated for 6 min in Krebs-Henseleit bicarbonate buffered saline (118 mmol/l NaCl, 4.8 mmol/l KCl, 25 mmol/l $NaHCO_3$; 1.2 mmol/l KH_2PO_4; 1.2 mmol/l $MgSO_4$; 2.5 mmol/l $CaCl_2$), containing 0.5 mmol/l myo-inositol, 5 mmol/l glucose, 5 mg/ml collagenase and 30 mg/ml bovine serum albumin, at 37°C and continuously gassed with 95% O_2:5% CO_2. Nerves were then washed three times in Krebs-Henseleit buffer and the epineurium removed. Under a dissecting microscope, the remaining perineurium of the major fascicles was pierced by a series of slits using fine watchmakers forceps. This procedure gave access to the endoneurial space for solutes in the bathing medium without damaging the axons and Schwann cells. The resulting endoneurial preparation from the left sciatic nerve was mounted between two suction electrodes and maintained in a thermoregulated organ bath at 37°C, containing continuously gassed (95% O_2:5% CO_2) Krebs-Henseleit buffer with 0.5 mmol/l myo-inositol and 5.0 mmol/l glucose. Electrical stimulation was applied to the proximal end of the preparation using an physiological stimulator (Model S88, Grass Instruments, Quincy, Mass., USA) and the resulting compound action potentials (CAPs) recorded on a digital storage oscilloscope (type 1421, Gould Instruments, Ilford, Essex, UK) via a differential pre-amplifier (DAM-5A, W-P Instruments Inc., New Haven, Conn., USA) with $\times$1000 gain and filters at 300 Hz and 30 KHz. Supramaximal stimulation (8–15 v) was applied for a 30 min stabilization period (0.5 Hz, 1.5 ms duration) with hard-copy recording plotted on an X-Y pen recorder every 10 min. This was done to ensure that the preparation was capable of propagating identical CAP's in the absence of hypoxia. After stabilization, stimulation was stopped and the bathing medium removed to be replaced with medium of the same composition but which had been previously gassed with a mixture of 8% O_2:5% CO_2:87% N_2. This gas mixture was also supplied to the organ bath, and stimulation recommenced for a further 40 min, with hard-copy recording at 10 min intervals.

Homogenate ATPase activity

Portions of endoneurial preparations derived from the right sciatic nerve were homogenised in 1 ml ice-cold buffer (130 mmol/l NaCl, 30 mmol/l KCl, 20 mmol/l Tris-Cl, 3 mmol/l $MgCl_2$ and 1 mmol/l EGTA at pH 7.6). The homogenate was centrifuged at low speed (180 $\times$ *g*, 5 min, 4°C) to remove large debris and protein content of the supernatant measured using a Coomassie blue-based spectrophotometric assay (Pierce Protein Assay Reagent, Pierce Chemical Co., Rockford, Ill., USA). Aliquots of each homogenate were also taken for assay of ATPase activity using a spectrophotometric procedure as described elsewhere [18] and Na^+/K^+-ATPase activity calculated as the fraction of total ATPase activity inhibited by the addition of 0.2 mmol/l ouabain to the reaction mixture. For each homogenate, triplicate measurements of ATPase activity (total and ouabain-insensitive) were made both in the presence and absence of 10 nmol/l porcine insulin (Sigma Chemical Co., St. Louis, Mo., USA) in the reaction mixture.

Sugar and polyol determination

Remaining portions of the right sciatic nerve were stored at −70°C until determination of sugar and polyol content by gas chromatography of the trimethylsilyl derivatives exactly as is described elsewhere [19].

Statistical analysis

The height of the first detected CAP, representing the fastest fibres, was measured on the hard copy obtained at $t = 0$ of the stabilisation and hypoxic periods. Subsequent measurements made at 10 min intervals were expressed as a percentage of these initial values.

Statistical comparison between groups was made using one-way ANOVA with comparison of group means by Duncan's multiple range test when the F ratio gave $p < 0.05$. Significance of the effects of insulin upon homogenate ATPase activities was assessed by paired *t*-test.

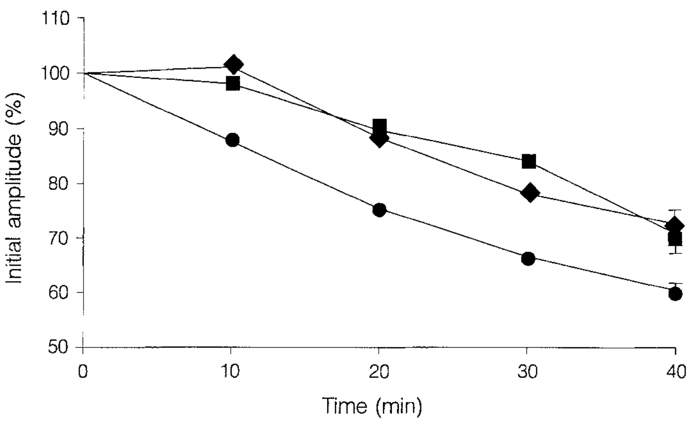

Fig. 1. Decline in the amplitude of the sciatic nerve compound action potential during 40 min hypoxia (8% O_2). The decline was greater in preparations from control rats than in those from either diabetic group. Error bars indicate the magnitude of the largest SEM for each regression. Key to symbols: circles = control rats, squares = untreated diabetic rats, diamonds = imirestat-treated diabetic rats

Results

Body weight, plasma glucose and MNCV

Untreated diabetic rats exhibited characteristic hyperglycaemia and reduced body weight compared to the control group (Table 1). Treatment of diabetic rats with imirestat was without effect upon these parameters. MNCV was significantly ($p < 0.05$) reduced in untreated diabetic rats compared to control values (Table 1). This deficit was completely prevented by aldose reductase inhibition.

Electrophysiological parameters during hypoxia in vitro

During the 30 min stabilisation period no preparations from any group showed any significant change in CAP amplitude (at $t = 30$ min, CAP amplitude as a % of the initial value [± 1 SD] was 99.4 ± 10.0 in control rats, 97.5 ± 8.7 in untreated diabetic rats and 99.9 ± 7.1 in imirestat-treated diabetic rats). CAP amplitude fell in all four groups during 40 min of hypoxia (Fig. 1), however, CAP amplitude declined much more rapidly in control nerves, such that there was a significant difference between controls and any other group after 10 min (all $p < 0.01$). This pattern was maintained during the course of hypoxia and at no point was there any significant difference between either of the diabetic groups.

Homogenate ATPase activity

Grouped data are shown in Table 2. Addition of insulin to homogenates made from control rat nerve caused a slight decrease in activity in the absence of ouabain (total or 'non-specific' ATPase activity). This effect was significant ($p < 0.01$) by comparison of basal activity with activity after the addition of insulin using paired t-tests. This 'horizontal' comparison is not shown in Table 2. Calculation of the ouabain-sensitive activity (Na^+/K^+-ATPase), by subtraction of the insensitive component, showed a stimulation by insulin of the Na^+/K^+-ATPase activity, which was always significant ($p < 0.01$) by paired testing in control homogenates.

In the absence of insulin, both untreated ($p < 0.01$) and imirestat-treated ($p < 0.05$) diabetic rats showed significantly reduced ouabain-insensitive ATPase activity compared to controls. Ouabain-sensitive (Na^+/K^+-ATPase) activity was similar in all groups.

In the presence of insulin, the ouabain-insensitive ATPase activity of the three groups showed a pattern similar to that described above. Thus, both untreated ($p < 0.01$) and aldose reductase-treated ($p < 0.05$) diabetic rats exhibited significantly reduced ouabain-insensitive ATPase activity. In the presence of insulin, both diabetic groups exhibited significantly reduced Na^+/K^+-ATPase activity compared to controls (untreated diabetics = $p < 0.01$, imirestat-treated = $p < 0.05$).

Insulin significantly reduced both total and oubain-insensitive ATPase activity in nerve homogenates of all groups (all $p < 0.01$ by paired t-test). Insulin was without effect upon homogenate Na^+/K^+-ATPase activity in the diabetic groups. However, the addition of insulin to homogenates from the control animals significantly increased Na^+/K^+-ATPase activity by 34% ($p < 0.05$ by paired t-test).

Table 1. Final body weight, plasma glucose and motor nerve conduction velocity (MNCV) in control rats, untreated diabetic rats and diabetic rats treated with the aldose reductase inhibitor, imirestat

	Body weight (g)	Plasma glucose (mmol/l)	MNCV (m/s)
Control (11)	432 ± 37^a	6.6 ± 0.9^a	51.0 ± 7.0^a
Diabetic (10)	318 ± 25^b	36.2 ± 7.3^b	42.5 ± 4.5^b
Diabetic + imirestat (9)	336 ± 41^b	31.0 ± 10.0^b	50.9 ± 8.8^a
Statistical significance	$^{a,b}p < 0.01$	$^{a,b}p < 0.01$	$^{a,b}p < 0.05$

Data are mean ± 1 SD. The number of animals per group is shown in parentheses. Statistical comparisons between groups by one-way ANOVA with Duncan's multiple range test

Table 2. ATPase activities (nmol ADP produced $\cdot h^{-1} \cdot$ mg protein^{-1}) of homogenates from sciatic nerves of control rats, untreated diabetic rats and diabetic rats treated with the aldose reductase inhibitor, imirestat, in the absence or presence of 10 nmol/l insulin

	Insulin absent		Insulin present	
	Ouabain-insensitive	Ouabain-sensitive	Ouabain-insensitive	Ouabain-sensitive
Control (11)	13933 ± 3124^a	2069 ± 851	11328 ± 2531^a	2766 ± 1039^a
Diabetic (10)	10198 ± 1889^b	1718 ± 420	8594 ± 1342^b	1458 ± 426^b
Diabetic + imirestat (8)	10452 ± 1074^b	1707 ± 522	9109 ± 1412^c	1870 ± 788^c
Statistical significance	$^{a,b}p < 0.01$		$^{a,b}p < 0.01$ $^{a,c}p < 0.05$	$^{a,b}p < 0.01$ $^{a,c}p < 0.05$

Data are mean ± 1 SD. The number of animals per group is shown in parentheses. Statistical comparisons between groups by one-way ANOVA with Duncan's multiple range test

Table 3. Nerve sugar and polyol content (nmol/mg dry weight) for control rats, untreated diabetic rats and diabetic rats treated with the aldose reductase inhibitor, imirestat

	Glucose	Sorbitol	Fructose	myo-inositol
Control (10)	2.6 ± 1.4^{a}	0.1 ± 0.3^{a}	1.8 ± 1.0^{a}	10.1 ± 1.9^{a}
Diabetic (9)	39.5 ± 11.8^{b}	7.2 ± 2.6^{b}	24.8 ± 6.8^{b}	7.6 ± 1.1^{b}
Diabetic + imirestat (7)	36.6 ± 16.1^{b}	0.04 ± 0.12^{a}	2.8 ± 1.9^{a}	9.1 ± 2.5
Statistical significance	$^{a,b}p < 0.01$	$^{a,b}p < 0.01$	$^{a,b}p < 0.01$	$^{a,b}p < 0.05$

Data are mean ± 1 SD. Numbers of animals are in parentheses. Statistical comparisons between groups were made using one-way ANOVA with Duncan's multiple range tests

Nerve sugars and polyols

Data are presented in Table 3. Sciatic nerves of untreated diabetic rats showed the classical increased content of glucose and the polyol pathway metabolites, sorbitol and fructose (all $p < 0.01$). myo-Inositol content was significantly decreased in diabetic rat nerve ($p < 0.01$). Imirestat treatment had no effect on glucose accumulation in diabetic nerve but sorbitol accumulation was prevented completely and fructose levels were very close to those seen in the nerves of non-diabetic rats (both $p < 0.01$ vs data from untreated diabetic rats). myo-Inositol content was increased toward control values but was not significantly different from either control or diabetic nerve values.

Discussion

In this study, treating diabetic rats with the aldose reductase inhibitor, imirestat, at a daily dose of 1 mg/kg completely prevented the accumulation of polyol pathway products and attenuated myo-inositol depletion in peripheral nerve. The drug prevented completely the deficit in sciatic MNCV in vivo. These findings are not new and a similar effect on the MNCV deficit has been reported using a variety of structurally unrelated aldose reductase inhibitors [20–22], although the much smaller dose employed in the present study may indicate a greater potency of imirestat.

Sciatic nerve preparations from diabetic rats exhibited a resistance to hypoxic conduction blockade in vitro. Preliminary studies have indicated that the time that control or diabetic preparations continue to function during hypoxia in vitro is not influenced by increasing the bathing medium glucose concentration to 25 mmol/l, and that the resistance to hypoxic conduction blockade in nerves from diabetic rats is reduced after intensive insulin therapy (unpublished data). These findings suggest that the continued function of diabetic nerve is not simply a consequence of increased substrate availability, and that streptozotocin toxicity per se does not underlie the phenomenon. The present study has shown that resistance to hypoxic conduction blockade in nerves from diabetic rats is unrelated to exaggerated polyol pathway flux and thus does not share a common aetiology with the MNCV disorder.

Previous studies have been unable to agree on the relationship between resistance to ischaemic/hypoxic conduction blockade and exaggerated polyol pathway flux. Where galactose feeding has been employed to increase flux through the first part of the polyol pathway, one study reported no effect upon the time for complete disappearance of caudal nerve CAP during ischaemia [8] whereas another demonstrated prolonged resistance of both caudal nerve CAP and muscle CAP to ischaemia [14]. However, consequences of galactose feeding that are secondary to polyol pathway flux, but absent from diabetic rats, such as increased water content leading to oedema, may restrict the extrapolation of any mechanisms from galactosaemic to diabetic rats [23].

Studies using diabetic rats have also been equivocal. Treating diabetic rats with aldose reductase inhibitors has been shown to be ineffective in preventing resistance to ischaemic conduction blockade where the time to complete disappearance of either caudal nerve or interosseous muscle CAP has been recorded [8, 15]. In contrast, the time taken for a 50% decline in muscle CAP was reduced in diabetic rats treated with an aldose reductase inhibitor [15]. It has been suggested that the discrepancy in the measurement end point contributes to inconsistencies in these studies, and that only the most rapidly failing nerve fibres (and thus those that contribute to the 50% decline value) are responsive to aldose reductase inhibitors [15]. However, studies involving the measurement of muscle CAP after sciatic nerve stimulation are complicated in their interpretation by the potential for effects on neuromuscular transmission and muscle function to contribute to measured changes. Given our lack of effect of imirestat on resistance to hypoxia, the effect on prolonged muscle CAP in diabetic rats reported in the above study may reflect correction of contributory factors other than nerve conduction per se or a specific action related to the aldose reductase inhibitor used and unrelated to inhibition of polyol pathway flux.

Homogenate maximal Na^{+}/K^{+}-ATPase activity was not reduced after 4 weeks of untreated streptozotocin diabetes. This is consistent with our previous study which described reduced maximal Na^{+}/K^{+}-ATPase activity in sciatic nerves after 8 but not 4 weeks of diabetes [24] but at variance with other investigators who have described deficient homogenate ATPase activity after 4 weeks of diabetes in streptozotocin-diabetic rats [25] using similar methods. These discrepancies have been discussed elsewhere [24]. The absence, in this study, of any deficit in maximal homogenate Na^{+}/K^{+}-ATPase activity after 4 weeks diabetes suggests that this cannot underlie the reduced MNCV or increased resistance to hypoxic conduction blockade measured in diabetic rats. However, it should be noted that the methodology does not allow extrapolation to changes in steady-state or stimulated nerve Na^{+} or K^{+} pumping in vivo.

The addition of 10 nmol/l insulin to endoneurial preparation homogenates from control rats resulted in an increase in maximal Na^{+}/K^{+}-ATPase activity. This is a novel finding in peripheral nerve and consistent with recent studies that have identified an insulin-stimulated Na^{+}/K^{+}-ATPase in rat forebrain synaptosomes [17] and several non-neuronal tissues [26, 27]. The nature of the interaction between insulin and Na^+/K^+-ATPase activity is at present unclear, but it is clear that insulin-stimulated Na^+/K^+-ATPase activity was absent from homogenates of diabetic rats. Judged empirically, it is unlikely that this disorder contributes to impaired MNCV in diabetic rats, simply because imirestat treatment restored the conduction velocity deficit without reinstating the capacity of insulin to stimulate homogenate Na^+/K^+-ATPase activity. Further work is needed to relate these two defects and this will require measurement of Na^+/K^+ pumping in intact nerve for more direct correlation with conduction.

In conclusion, this study has demonstrated that two functional disorders of peripheral nerve in acute diabetes, namely impaired MNCV and increased resistance to hypoxic conduction blockade, do not share a common aetiology. Sciatic nerve homogenates from diabetic rats also exhibit a reduction in insulin-sensitive maximal Na^+/K^+-ATPase activity, which was also resistant to aldose reductase inhibition.

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