

## Insulin partially reverses deficits in peripheral nerve blood flow and conduction in experimental diabetes

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### Abstract

Decreased nerve blood flow may be a pathogenetic factor in diabetic neuropathy. Previously it was shown that insulin treatment, commenced at the onset of streptozotocin-diabetes, prevents the development of a nerve blood flow deficit in the diabetic rat. The present study sought to determine the effect of short-term (one month) and acute (one hour) insulin reversal treatment on nerve blood flow deficits in streptozotocin-diabetes. Sciatic nerve blood flow was assessed using laser Doppler flowmetry. Treatment was initiated after one month of diabetes. One month of reversal insulin treatment ameliorated nerve laser Doppler flux (NDF) deficits; in untreated diabetic rats NDF was 51% of that in control animals ( $P < 0.01$ ), in insulin-treated diabetic rats NDF was 85% of control values ( $P < 0.01$  vs. untreated diabetic,  $P < 0.05$  vs. control). In association with blood flow increases, we found a significant amelioration of motor ( $P < 0.05$  vs. untreated diabetic) and sensory ( $P < 0.01$  vs. untreated diabetic) nerve conduction velocities but not of exaggerated resistance to hypoxic conduction block. Insulin partially reversed hyperglycaemia and sciatic nerve polyol and sugar levels. In a second experiment, in rats with one month of diabetes, acute infusion of insulin led to a 47% ( $P < 0.001$  vs. pre-insulin values) reduction of plasma glucose. This fall in plasma glucose was accompanied by a 38% ( $P < 0.05$  vs. pre-insulin values) increase in NDF. Sensory nerve conduction velocity was marginally increased (6%,  $P < 0.05$  vs. pre-insulin values) after insulin infusion, but motor conduction velocity was not. The data indicate that insulin can partially reverse deficits in nerve blood flow and conduction in diabetic rats.

**Keywords:** Diabetic neuropathy; Nerve blood flow; Laser Doppler flowmetry; Nerve conduction; Streptozotocin-diabetes; Insulin

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### 1. Introduction

Early peripheral neurological dysfunction in diabetic rats is characterised by reduced nerve conduction velocity (Eliasson, 1964) and increased resistance to hypoxic conduction block (Seneviratne and Peiris, 1969); in the long term, structural changes also develop (Chopra et al., 1977; Jakobsen, 1978). The pathogenesis of diabetic neuropathy has been studied extensively and metabolic and vascular mechanisms have been proposed to explain the defects described (Greene and Lattimer, 1983; Tuck et al., 1984). The metabolic hypothesis concerns the effects of increased glucose flux through the polyol pathway in diabetes with resultant accumulation of sorbitol and fructose and depletion of *myo*-inositol in peripheral nerve. Support for a vascular mechanism is centred on clinical and experimen-

tal studies, showing reduced endoneurial oxygen tension (Tuck et al., 1984; Newrick et al., 1986) and impaired nerve blood flow (Tuck et al., 1984; Cameron et al., 1991; Stevens et al., 1993; Kappelle et al., 1993; Tesfaye et al., 1993). Furthermore, treatment of diabetic rats with vasoactive agents can ameliorate both nerve blood flow and conduction velocity deficits (for example, Stevens et al., 1993; Kappelle et al., 1993, 1994; Yasuda et al., 1989; Maxfield et al., 1993; Cameron et al., 1994b; Karasu et al., 1995; Stevens and Tomlinson, 1995). The development of diabetic neuropathy in humans is associated with diabetes duration and metabolic control (Pirart, 1978a,b) and can be attenuated by intensive insulin treatment (The Diabetes Control and Complications Study Group, 1993; Amthor et al., 1994). In a previous study we showed that short-term treatment with insulin prevented peripheral nerve ischaemia in streptozotocin-diabetic rats (Stevens et al., 1994a). The present study sought to determine whether treatment with insulin can reverse existing nerve ischaemia

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in diabetic rats. Treatment with insulin was initiated after one month of diabetes, at which time there is a marked reduction in sciatic nerve blood flow as determined with laser Doppler flowmetry (Kappelle et al., 1993; Stevens et al., 1994a). The effects of insulin were evaluated in two experiments. Experiment 1 studied the effects of short-term (1 month) insulin treatment, while Experiment 2 determined the effect of acute insulin administration (1 h). Indices of nerve blood flow (nerve laser Doppler flux), nerve conduction (motor and sensory conduction velocity and resistance to hypoxic conduction block) and nerve metabolic status (sugar and polyol levels) were measured.

## 2. Materials and methods

### 2.1. Experimental organisation

The study consisted of two separate experiments, both of which used male Wistar rats (starting weight 250–300 g, 8–11 weeks of age; Charles River, Margate, UK). Rats were housed on sawdust and maintained on a 12–12 h light–dark cycle. All rats were given food and water ad libitum and were weighed weekly. Diabetes was induced by a single intraperitoneal injection of streptozotocin at a dose of 65 mg kg<sup>-1</sup> body weight (ICI Pharmaceuticals, Macclesfield, UK) dissolved in 0.9% saline. Rats were fasted overnight prior to the STZ administration. Two days after the injection blood glucose was determined in blood samples, obtained by tail prick, by strip-operated reflectance photometry (Reflolux S, Boehringer-Mannheim, Mannheim, Germany). Blood glucose levels were > 15 mmol l<sup>-1</sup> in all streptozotocin-injected animals.

In Experiment 1, the short-term insulin reversal study, three groups of rats were used; a non-treated, non-diabetic and age-matched control group ( $n = 9$ ), a non-treated diabetic group ( $n = 8$ ) and an insulin-treated diabetic group ( $n = 8$ ). Insulin treatment was via two subcutaneous sustained release insulin implants per rat (Linplant; Møllegaard, Ejby, Denmark; release per implant 2 IU day<sup>-1</sup> for > 40 days; (Kappelle et al., 1994; Stevens et al., 1994a)) given one month after the induction of diabetes. Treatment was continued for one month, and treatment efficacy was determined weekly by measuring blood glucose in blood samples, obtained by tail prick, by strip-operated reflectance photometry. At the end of the treatment period, nerve laser Doppler flux and nerve conduction velocities were determined in the left sciatic nerve. The left nerve was then used to determine resistance to hypoxic conduction block in vitro. The right sciatic nerve was removed and stored at -70°C until later determination of nerve sugar and polyol content. Finally, blood was collected by cardiac puncture for the determination of plasma glucose (GOD-PERID test-kit, Boehringer-Mannheim) and glycosylated haemoglobin levels (HbA<sub>1</sub> test-kit, Sigma, Poole, UK) in order to confirm the efficacy of the insulin regime in treated rats.

In Experiment 2, the acute insulin administration study, two groups of rats were used: a non-diabetic age-matched control group ( $n = 5$ ) and a diabetic group ( $n = 7$ ). Both groups were left untreated for one month. After measurement of baseline values of nerve Doppler flux and conduction velocities, an arterial blood sample was taken for determination of plasma glucose content. In diabetic animals, insulin (Human Actrapid; Novo Nordisk, Denmark via Novo Nordisk, Crawley, UK), dissolved in Haemaccel (Plasma substitute; Behringwerk, Marburg, Germany, supplied via Hoechst UK, Hounslow, UK) at 4 IU ml<sup>-1</sup>, was injected via the jugular vein. An initial bolus of insulin (2 IU kg<sup>-1</sup> body weight) was followed by maintenance infusion (rate 750 µl h<sup>-1</sup>; 10 IU kg<sup>-1</sup> h<sup>-1</sup>). This regime was designed to deliver a total of 3–4 IU insulin per rat, which was equivalent to the daily dose for a treated rat from the first study. One hour after the administration of the insulin bolus, nerve Doppler flux and conduction velocities were again determined and a second blood sample was taken for later measurement of plasma glucose content. In the control rats a similar procedure was followed to determine the effect of the experimental procedure on nerve and cardiovascular function but, instead of administering insulin, Haemaccel alone was infused.

### 2.2. Nerve laser Doppler flux

Nerve laser Doppler flux was determined in the sciatic nerve using laser Doppler flowmetry as described previously (Stevens et al., 1994a). In short, animals were anaesthetised initially with halothane (4% in O<sub>2</sub> for induction, 2–2.5% in O<sub>2</sub> for maintenance) and the left jugular vein was cannulated. The rats were then transferred to intravenous Saffan (alphaxalone 9 mg ml<sup>-1</sup> and alphadolone 3 mg ml<sup>-1</sup>; Pitman-Moore, Uxbridge, UK). Anaesthesia was maintained by an initial bolus of 1.5 ml kg<sup>-1</sup> of a 1:3 dilution of the manufacturer's solution with 0.9% saline. This was followed by infusion with a 1:6 dilution of Saffan in 0.9% saline at a rate of 10 ml kg<sup>-1</sup> h<sup>-1</sup> throughout the experiment. The carotid artery was cannulated for the measurement of systemic arterial pressure and heart rate.

Via a small incision in the left flank the sciatic nerve was exposed. A fibre optic probe (Type P4; tip diameter 0.85 mm; Moor Instruments, Axminster, UK) was positioned so that it was just in contact with the nerve. The Doppler signal was registered on the flow-monitor (Type MBF3D; Moor Instruments). Both blood pressure and nerve laser Doppler flux signals were analysed on a Macintosh personal computer (AD Instruments, London, UK). Values for cardiovascular variables were averaged over a 1-min recording period. Mean values for nerve Doppler flux were calculated from 10 consecutive measurements along the exposed portion of nerve. Body temperature was maintained at 37.5°C throughout the procedure and near-

nerve temperature was 36–37°C during all measurements. Sciatic nerve vascular conductance was calculated by dividing nerve Doppler flux by mean arterial pressure.

### 2.3. Motor and sensory nerve conduction velocity

Immediately after nerve Doppler flux measurements, motor and sensory nerve conduction velocities were determined by a method adapted from that described by De Koning and Gispen (1987). The left sciatic and tibial nerve were stimulated at the sciatic notch and ankle respectively, by means of monopolar needle electrodes. The anode was placed in the lower back musculature. The stimulus was a 150  $\mu$ s unipolar pulse generated by a Neurolog NL 301 Pulse generator (Neurolog, Digitimer, Hertfordshire, UK) and delivered through a Neurolog NL 800 stimulus isolator. The stimulation voltage was 10 V. Responses were recorded by two monopolar needle electrodes, one inserted in the dorsal interosseus muscle between digits III and IV and the other in the plantar muscles of the foot. The responses were amplified via a Neurolog preamplifier and digitized and analysed on a Macintosh personal computer. Two responses were recorded: the short latency M-response, due to direct stimulation of the  $\alpha$ -motor fibres, and the long latency H-response due to stimulation of sensory Ia-fibers, which monosynaptically excite motoneurons in the spinal cord. Near-nerve temperature was 37°C during measurements. Motor and sensory conduction velocities were calculated from the latencies of these responses and the distance between the stimulation points as determined with callipers.

### 2.4. Resistance to hypoxic conduction block

Resistance to hypoxic conduction block was determined by a previously described method (Stevens et al., 1993; Calcutt et al., 1991). In short, the left sciatic nerve was dissected rapidly and incubated in an organ bath in Krebs-Henseleit bicarbonate buffered saline (118 mmol l<sup>-1</sup> NaCl; 4.8 mmol l<sup>-1</sup> KCl; 25 mmol l<sup>-1</sup> NaHCO<sub>3</sub>; 1.2 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>; 1.2 mmol l<sup>-1</sup> MgSO<sub>4</sub>; 2.5 mmol l<sup>-1</sup> CaCl<sub>2</sub>) containing 0.5 mmol l<sup>-1</sup> *myo*-inositol and 5.0 mmol l<sup>-1</sup> D-glucose at 37°C and continuously gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The proximal end of the nerve was stimulated at a frequency of 0.5 Hz, with a supramaximal

stimulus of 10 V, duration 100  $\mu$ s. The resulting compound action potentials were recorded from the distal end of the nerve via a digital storage oscilloscope (Type 1421; Gould Instruments, Ilford, UK). Every 10 min, recordings were stored on a personal computer. After an initial stabilisation period of 30 min the buffer was replaced with that of the same composition which had been previously gassed with a mixture of 8% O<sub>2</sub>, 5% CO<sub>2</sub> and 87% N<sub>2</sub>. This gas mixture was supplied to the organ bath for a further 50 min, with recordings at 10-min intervals.

### 2.5. Measurement of nerve sugar and polyol content

Glucose, fructose, sorbitol and *myo*-inositol levels were determined in the right sciatic nerve by gas chromatography of the trimethylsilyl derivatives, using  $\alpha$ -methyl mannoside as an internal standard, as described elsewhere (Mayer and Tomlinson, 1983).

### 2.6. Data analysis

Data are expressed as mean  $\pm$  1 SD. For Experiment 1, the short-term insulin reversal study, statistical analyses were carried out by one-way analysis of variance. Where the F ratio gave  $P < 0.05$  and there was homogeneity of variances (Levene test,  $P > 0.05$ ), group means were compared using Duncan's multiple range tests. The resistance to hypoxic conduction block data were compared with analysis of variance for repeated measurements and, for values after 50 min of hypoxia, one-way analysis of variance and Duncan's multiple range tests. Correlations are expressed as the Pearson product moment coefficient ( $r$ ). For Experiment 2, the acute insulin administration study, comparisons of data before and after Haemaccel or insulin administration were carried out by Student's paired  $t$ -tests for control and diabetic groups, respectively.

## 3. Results

### 3.1. Experiment 1 (short-term insulin reversal study)

#### 3.1.1. Animals (Table 1)

Untreated diabetic rats had significantly reduced final body weights, together with raised levels of plasma glu-

Table 1  
Experiment 1 (insulin reversal study): body weight, final plasma glucose and glycosylated haemoglobin data

	<i>n</i>	Body weight (g)		Final plasma glucose (mmol l <sup>-1</sup> )	Glycosylated haemoglobin (%)
		Initial	Final		
Control	9	379 $\pm$ 11	535 $\pm$ 66 <sup>x</sup>	8.5 $\pm$ 1.5 <sup>x</sup>	4.4 $\pm$ 0.8 <sup>x</sup>
Untreated diabetic	8	382 $\pm$ 14	293 $\pm$ 54 <sup>y,X</sup>	42.6 $\pm$ 9.7 <sup>y,X</sup>	9.5 $\pm$ 1.7 <sup>y</sup>
Insulin-treated diabetic	8	378 $\pm$ 19	448 $\pm$ 40 <sup>y,Y</sup>	20.3 $\pm$ 7.1 <sup>y,Y</sup>	6.4 $\pm$ 2.6 <sup>x</sup>

Data are mean  $\pm$  1 S.D. and were analysed by one-way analysis of variance with Duncan's multiple range tests;  $P < 0.01$  (x vs. y, X vs. Y). For statistical analysis, plasma glucose data was transformed to natural logarithms to achieve homogeneity of variances.

cose and glycosylated haemoglobin, compared to control animals. Reversal treatment with insulin attenuated these diabetes-induced anomalies. Blood glucose concentrations were determined weekly in blood samples from insulin treated animals. Before implantation blood glucose values were  $> 30 \text{ mmol l}^{-1}$  (detection limit Refloflux) in seven animals, and  $27.1 \text{ mmol l}^{-1}$  in one. Mean values were  $22.3 \pm 6.0$ ,  $13.3 \pm 7.2$ ,  $8.9 \pm 8.0$  and  $8.6 \pm 6.2 \text{ mmol l}^{-1}$  at three days, one, two and three weeks after implantation, respectively. Final blood glucose was  $18.3 \pm 6.3 \text{ mmol l}^{-1}$ , which corresponded with a plasma glucose of  $20.3 \pm 7.1 \text{ mmol l}^{-1}$  ( $42.6 \pm 9.7$  in untreated diabetic rats). In the two groups of diabetic rats, there was a good correlation between plasma glucose and glycosylated haemoglobin values ( $r = 0.74$ ,  $P < 0.001$ ).

### 3.1.2. Sciatic nerve Doppler flux, nerve vascular conductance and cardiovascular variables (Fig. 1, Table 2)

Nerve Doppler flux in untreated diabetic rats ( $188 \pm 55$  arbitrary units) was 51% ( $P < 0.01$ ) of that for control animals ( $370 \pm 67$  arbitrary units). Values for insulin-treated diabetic rats ( $309 \pm 51$  arbitrary units) were significantly different from those of both the untreated control ( $P < 0.05$ ) and diabetic ( $P < 0.01$ ) groups. Mean and diastolic arterial pressures were similar and not significantly different between experimental groups, while systolic pressures were significantly lower in rats of the untreated diabetic group compared to those of control animals ( $P < 0.01$ ). Heart rates were also significantly lower for untreated diabetic rats compared to control animals ( $P < 0.05$ ), while those for diabetic rats which received insulin did not differ from those of either the untreated diabetic or control groups. Calculated sciatic nerve vascular conductances were significantly lower in untreated diabetic animals compared to either control ( $P < 0.01$ ) or insulin-treated diabetic rats ( $P < 0.01$ ).

### 3.1.3. Motor and sensory nerve conduction velocity (Fig. 1)

Untreated diabetic animals had both significantly ( $P < 0.01$ ) reduced sciatic motor ( $44.6 \pm 3.0 \text{ m s}^{-1}$ ) and sensory ( $44.0 \pm 2.1 \text{ m s}^{-1}$ ) nerve conduction velocities compared to control animals ( $50.6 \pm 1.8$  and  $52.7 \pm 4.2 \text{ m s}^{-1}$ , respectively). The reduction in motor conduction velocity was attenuated in diabetic rats treated with insulin ( $47.3 \pm 1.3 \text{ m s}^{-1}$ ,  $P < 0.01$  vs. control and  $P < 0.05$  vs. un-

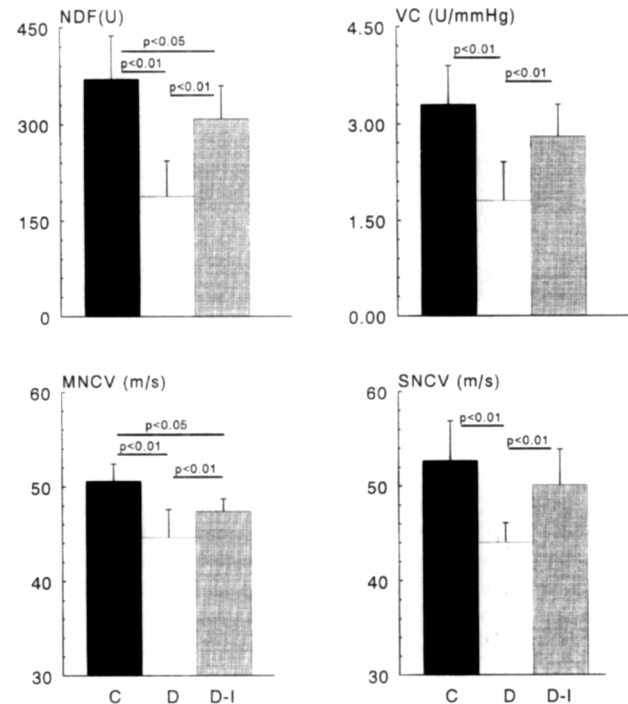


Fig. 1. Sciatic nerve laser Doppler flux (NDF), vascular conductance (VC) and motor and sensory nerve conduction velocities (MNCV and SNCV) for control (C), untreated diabetic (D) and insulin-treated diabetic (D-I) rats from Experiment 1, the short-term insulin reversal study. Data are mean  $\pm 1$  S.D. and were analysed by one-way analysis of variance and Duncan's multiple range tests.

treated diabetic) and that in sensory conduction was reversed by insulin treatment ( $50.1 \pm 3.8 \text{ m s}^{-1}$ , not significantly different to control and  $P < 0.01$  vs. untreated diabetic). When the values for all animals were considered, those for nerve Doppler flux and vascular conductance correlated with motor ( $r = 0.67$ ,  $P < 0.0005$  and  $r = 0.69$ ,  $P < 0.0005$ , respectively) and, to a lesser extent, with sensory ( $r = 0.45$ ,  $P < 0.02$  and  $r = 0.42$ ,  $P < 0.05$ , respectively) nerve conduction velocity.

### 3.1.4. In vitro sciatic nerve resistance to hypoxia (Fig. 2)

Analysis of variance for repeated measurements revealed significant differences between nerve compound action potential values for the three experimental groups over the 10 to 50 min of hypoxia ( $P < 0.005$ ). In the untreated diabetic rats, decreases in nerve compound action potential amplitudes during enforced hypoxia were

Table 2

Experiment 1 (insulin reversal study): cardiovascular data

	Systemic arterial pressures (mmHg)			Heart rate (beats $\text{min}^{-1}$ )
	Systolic	Diastolic	Mean	
Control	$136 \pm 9^x$	$98 \pm 9$	$113 \pm 9$	$365 \pm 54^a$
Untreated diabetic	$123 \pm 11^y$	$92 \pm 8$	$107 \pm 9$	$320 \pm 44^b$
Insulin-treated diabetic	$130 \pm 5$	$95 \pm 6$	$110 \pm 6$	$338 \pm 17$

Data are mean  $\pm 1$  S.D. and were analysed by one-way analysis of variance with Duncan's multiple range tests;  $P < 0.01$  (x vs. y) and  $P < 0.05$  (a vs. b).

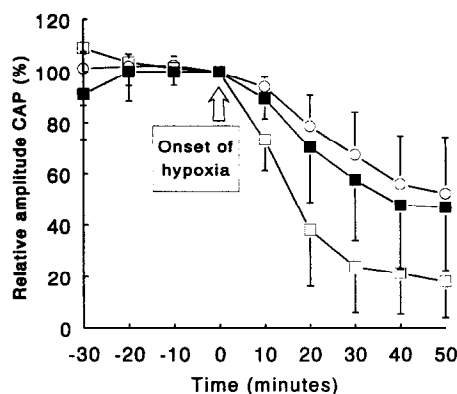


Fig. 2. Sciatic nerve in vitro resistance to hypoxic conduction block for control ( $\square$ ), untreated diabetic ( $\circ$ ) and insulin-treated diabetics rats ( $\blacksquare$ ) from Experiment 1, the short-term insulin reversal study. Compound action potentials (CAP) are expressed as percentage of that at 0 min. Data are mean  $\pm$  1 S.D. One-way analysis of variance for repeated measurements revealed significant differences between the three experimental groups over the hypoxic period (time = 10–50 min,  $P < 0.005$ ). One-way analysis of variance and Duncan's multiple range tests at the end of hypoxia (time = 50 min) showed significant differences between control and both untreated and insulin-treated diabetic rats ( $P < 0.01$  and  $P < 0.05$  respectively). There were no significant differences between untreated and insulin-treated diabetic rats.

markedly less than those recorded for nerves from control animals (% of remaining amplitude after 50 min of hypoxia  $52.4 \pm 22.0$  in untreated diabetic and  $18.1 \pm 19.9$  in control,  $P < 0.01$ ). These changes were also similar for diabetic animals which were treated with insulin ( $46.9 \pm 24.4\%$ ,  $P < 0.05$  vs. control). The values correlated neither with parameters of metabolic control, such as glycosylated haemoglobin, plasma glucose, nerve glucose, nerve myo-inositol or nerve sorbitol, nor with conduction velocities or nerve Doppler flux.

### 3.1.5. Nerve sugar and polyol content (Table 3)

Sciatic nerve glucose, fructose and sorbitol content were increased several-fold in untreated diabetic rats as compared to those for control rats. The increases were attenuated by insulin treatment, but were not restored to control levels. Myo-inositol levels were significantly decreased in untreated diabetic rats compared to control rats. The reduction in myo-inositol was also partially restored by insulin treatment.

Table 3

Experiment 1 (insulin reversal study): sugar and polyol data

	Sciatic nerve sugar and polyol content (nmol mg <sup>-1</sup> dry tissue weight)			
	Glucose	Fructose	Sorbitol	Myo-inositol
Control	$2.65 \pm 0.86^x$	$3.11 \pm 0.84^x$	$0.69 \pm 0.28^x$	$6.73 \pm 0.60^{x,a}$
Untreated diabetic	$24.74 \pm 5.85^{y,a}$	$14.50 \pm 3.33^{y,a}$	$8.82 \pm 3.21^{y,X}$	$4.86 \pm 0.84^{y,a}$
Insulin-treated diabetic	$9.53 \pm 3.15^{y,b}$	$9.43 \pm 1.39^{y,b}$	$3.77 \pm 1.59^{y,Y}$	$5.91 \pm 0.71^b$

Data are mean  $\pm$  1 S.D. and were analysed by one-way analysis of variance with Duncan's multiple range tests;  $P < 0.01$  (x vs. y, X vs. Y) and  $P < 0.05$  (a vs. b).

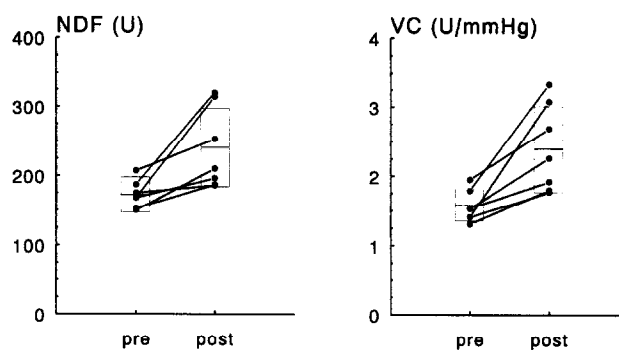


Fig. 3. Sciatic nerve laser Doppler flux (NDF) and vascular conductance (VC) data for diabetic rats before and after administration of insulin in Experiment 2, the acute insulin administration study. Data are individual data points for each rat and boxes represent mean  $\pm$  1 S.D.; statistical analyses are described in text.

### 3.2. Experiment 2 (acute insulin administration study)

#### 3.2.1. Animals (Table 4)

As for Experiment 1, untreated diabetic rats had markedly reduced body weights ( $279 \pm 17$  g) compared to control animals ( $521 \pm 40$  g) at the end of the protocol and were hyperglycaemic. In diabetic rats, after 1 h of insulin administration, plasma glucose values decreased significantly ( $P < 0.001$ ) by approximately 47%. In control rats, 1 h of Haemaccel administration had no significant effect on plasma glucose levels.

#### 3.2.2. Sciatic nerve laser Doppler flux and vascular conductance and cardiovascular data (Fig. 3 and Table 4)

Prior to Haemaccel or insulin administration, nerve Doppler flux for diabetic rats was 59% of that of control animals. Heart rate and arterial pressures were lower in control and diabetic rats before either Haemaccel or insulin administration.

For control rats, Haemaccel administration had no effect on either systemic cardiovascular variables (Table 4) or nerve Doppler flux (before  $292 \pm 61$  arbitrary units, after  $283 \pm 49$  arbitrary units). For diabetic rats, diastolic and mean arterial pressure decreased significantly ( $P < 0.001$ ,  $P < 0.01$  respectively) after insulin administration (Table 4), while nerve Doppler flux values increased significantly ( $P < 0.05$ ) post-insulin (Fig. 3). Changes in

Table 4

Experiment 2 (acute insulin administration study): plasma glucose and cardiovascular data

	Plasma glucose (mmol l <sup>-1</sup> )	Systemic arterial pressures (mmHg)			Heart rate (beats min <sup>-1</sup> )
		Systolic	Diastolic	Mean	
Control-pre	5.0 ± 0.9	141 ± 6	108 ± 4	124 ± 4	408 ± 15
Control-post	4.5 ± 0.7	145 ± 7	108 ± 6	125 ± 7	404 ± 17
Diabetic-pre	33.9 ± 1.9 <sup>x</sup>	126 ± 5	94 ± 3 <sup>x</sup>	111 ± 3 <sup>x</sup>	354 ± 19
Diabetic-post	18.2 ± 2.7 <sup>y</sup>	119 ± 4	78 ± 3 <sup>y</sup>	100 ± 3 <sup>y</sup>	358 ± 20

Data are mean ± 1 S.E.M. and were analysed with a two-tailed *t*-test for paired samples; *P* < 0.01 (x vs. y), *P* < 0.001 (X vs. Y).

nerve Doppler flux correlated with those in plasma glucose in diabetic animals ( $r = -0.76$ ,  $P = 0.046$ ). As for Experiment 1 and prior to administration of Haemaccel or insulin, calculated sciatic nerve vascular conductances were lower in diabetic animals compared to those of control rats. Haemaccel administration had no effect on sciatic nerve vascular conductance in control rats (before  $2.37 \pm 0.53$  arbitrary units mmHg<sup>-1</sup>, after  $2.33 \pm 0.62$  arbitrary units mmHg<sup>-1</sup>), but for diabetic animals values were significantly ( $P < 0.05$ ) increased after insulin administration (Fig. 3).

### 3.2.3. Motor and sensory nerve conduction velocity

As for Experiment 1 and pre-infusion of Haemaccel or insulin, both motor and sensory nerve conduction velocities were lower in diabetic animals ( $43.4 \pm 4.3$  and  $45.3 \pm 4.8$  m s<sup>-1</sup>, respectively) compared to those of rats in the control group ( $51.4 \pm 0.67$  and  $54.8 \pm 4.0$  m s<sup>-1</sup>, respectively). For motor nerve conduction velocity, neither Haemaccel nor insulin administration affected values for control ( $52.4 \pm 2.0$  m s<sup>-1</sup>) or diabetic ( $44.0 \pm 2.2$  m s<sup>-1</sup>) animals, respectively. Sensory nerve conduction velocity in control rats was also unaffected by Haemaccel administration ( $55.1 \pm 4.6$  m s<sup>-1</sup>). For diabetic rats, sensory conduction was significantly ( $P < 0.05$ ) increased after insulin administration ( $50.0 \pm 5.2$  m s<sup>-1</sup>). These changes correlated neither with those in plasma glucose or nerve Doppler flux.

## 4. Discussion

Untreated diabetic rats in both Experiments 1 and 2 showed hyperglycaemia and decreased weight gain compared to control animals. Effectiveness of the short-term reversal insulin treatment in Experiment 1 was evidenced by partial restoration of plasma glucose and glycosylated haemoglobin levels and body weight by the end of the protocol. Blood glucose levels appeared to increase in the final week of the experiment relative to the initial weeks of insulin treatment. Although this might indicate that glycaemic control was in decline at the end of the study, the level of glycaemic control was still superior to that in the untreated diabetic rats. In previous studies the insulin implants provided a constant level of glycaemic control for

one month or more (Kappelle et al., 1994; Stevens et al., 1994a). Possibly the nature of the present study, reversal of hyperglycaemia rather than prevention, plays a role in the reduced responsiveness to insulin. In STZ-diabetic rats insulin resistance has been detected 48 h or more after the STZ injection, affecting both glucose production and glucose utilisation (Lisato et al., 1992; Koopmans et al., 1991). Possibly the diminished response to insulin in Experiment 1 is related to the development of insulin resistance during the first month of untreated hyperglycaemia.

Untreated diabetic rats in Experiment 1 exhibited decreased motor and sensory nerve conduction velocities, increased nerve resistance to hypoxia, accumulation of nerve glucose, fructose and sorbitol and associated depletion of nerve *myo*-inositol, all compared to values for control rats. Moreover, nerve laser Doppler flux was reduced in the untreated diabetic rats, relative to control animals, in both experiments, confirming earlier reports of decreases in experimental diabetes (Stevens et al., 1993, 1994a; Kappelle et al., 1993; Yasuda et al., 1989; Maxfield et al., 1993; Karasu et al., 1995; Stevens and Tomlinson, 1995; Cameron et al., 1994a; Calcutt et al., 1994). Reductions of nerve blood flow in diabetic rats have also been found with other techniques, such as hydrogen clearance (Tuck et al., 1984; Cameron et al., 1991). The limitations of the laser Doppler technique in the measurement of nerve blood flow have been discussed previously (Stevens et al., 1993, 1994a; Kappelle et al., 1993). Although laser Doppler flowmetry cannot distinguish between endoneurial, perineurial and epineurial blood flow, there is a relationship with nerve blood flow as determined with [<sup>14</sup>C]iodoantipyrine and the slow component of the hydrogen clearance curve (Rundquist et al., 1985; Stevens et al., 1994b; Takeuchi and Low, 1987). Moreover, the technique provides highly reproducible data; in particular, the proportional deficit in diabetic rats compared to controls is similar in many studies from different laboratories (see above references). In diabetic rats, there is accumulating evidence to support a role for decreased nerve perfusion in the development of nerve conduction velocity deficits (see Introduction). Whether there is a role for nerve blood flow reduction in the pathogenesis of human diabetic neuropathy remains to be seen. Currently, only indirect evidence for nerve blood flow reductions in diabetic patients is available; endoneurial oxygen tension is reduced (Newrick

et al., 1986), fluorescein angiography of the sural nerve shows impairments (Tesfaye et al., 1993) and there are microvascular abnormalities (Tesfaye et al., 1994).

In Experiment 1, short-term reversal insulin treatment reversed diabetes-induced reductions in both motor and sensory conduction velocities and in nerve Doppler flux. These effects were associated with an improvement in glycaemic control and in nerve metabolic status, as evidenced by reductions in nerve glucose, fructose and sorbitol levels and increases in nerve *myo*-inositol levels compared to untreated diabetic rats. The enhancement of nerve Doppler flux in diabetic rats by insulin treatment occurred in the absence of systemic cardiovascular effects. Insulin treatment was initiated after one month of diabetes, at which time there is a 30–50% reduction in nerve Doppler flux (Experiment 2; Kappelle et al., 1993; Stevens et al., 1994a). After one month of insulin treatment nerve Doppler flux was restored to 84% of control levels. The finding that the recovery was incomplete may be due to imperfect glycaemic control or to some irreversible component of the diabetic deficit yet to be determined. These findings confirm and extend our previous insulin prevention study (Stevens et al., 1994a).

The increased resistance to hypoxic conduction block was the only functional variable for diabetic nerve which did not improve significantly with the short-term reversal insulin treatment in Experiment 1. In a previous study an intensive insulin treatment regimen partially prevented the development of this anomaly in streptozotocin-diabetic rats (Calcutt et al., 1991). The differences in these results may reflect those in the treatment protocols (prevention vs. reversal; twice-daily injections vs. slow-release implants). Exaggerated resistance to hypoxia has been reported in both diabetic patients and animals (Seneviratne and Peiris, 1969; Steiness, 1959; Gregersen, 1969). In the former, the magnitude of resistance appears to be related to the degree of medium-term metabolic control, rather than the presence or absence of neuropathy (Price et al., 1987). It has been suggested that increased resistance to hypoxia results from increased availability of substrate for anaerobic energy production in the diabetic nerve (Shirabe et al., 1988; Parry and Kohzu, 1990; Schneider et al., 1993). We cannot confirm this suggestion since values in this experiment did not correlate with either those for plasma or nerve glucose in diabetic rats. Others have suggested that increased resistance to hypoxia reflects an adaptation of the diabetic nerve to prolonged incipient hypoxia as a result of decreased nerve blood flow (Low et al., 1985, 1986a). This suggestion is supported by the demonstration of similar abnormalities in patients with lung or cardiac disease leading to hypoxaemia (Hampton et al., 1989; Masson et al., 1988) and in rats subjected to chronic hypoxia (Low et al., 1986b). The present study, however, demonstrates an improvement of nerve perfusion in the absence of an effect on resistance to hypoxia, questioning reduced blood flow as the single causal factor for this phenomenon in diabetic

nerve. The finding that insulin ameliorated nerve conduction velocity deficits independently from resistance to hypoxia is in accordance with earlier reports of differential effects of pharmacotherapy on these two measurements (Carrington et al., 1991; Robertson et al., 1992), suggesting that the electrophysiological variables do not share a common aetiology. Based upon the previous and present studies, the relevance of an enhanced peripheral nerve resistance to hypoxic conduction block in vitro is not established, so the value of such measurements for the assessment of treatment efficacy in diabetic nerve dysfunction may be questionable.

In Experiment 2, the effect of reducing plasma glucose concentration by acute insulin administration in diabetic rats was examined. Insulin administration had both systemic and local haemodynamic effects. The finding that diastolic and mean arterial pressure decreased upon insulin administration agrees with parallel alterations in blood pressure following acute, but not chronic, increases in plasma insulin in humans (Anderson et al., 1991; Baron, 1994). These changes were independent of alterations in plasma glucose. In our study, despite the decreased systemic arterial pressure, nerve laser Doppler flux increased upon insulin administration. This was reflected in the greater rise in sciatic nerve vascular conductance (54%) than in nerve Doppler flux (38%). The opposing effects on perfusion pressure and Doppler flux indicate that there is a local effect of the increase in insulin and/or the reduction in blood glucose on the nerve vasculature. Insulin can decrease vascular resistance in skeletal muscle beds independently of an effect on plasma glucose (Baron and Brechtel, 1993), it may well have a direct effect on nerve vasculature. In a recent study Kihara et al. addressed this issue by studying the effect of acute insulin infusion on endoneurial oxygen tension in non-diabetic rats (Kihara et al., 1994). They demonstrated that insulin actually decreased endoneurial oxygen tension in these rats, which they attributed to increased arteriovenous shunting in response to insulin administration. These findings are in apparent conflict with our present results, and it could be argued that the observed increase in nerve Doppler flux in response to acute insulin administration only reflected this increase in shunting. However, a hypoxic effect of exogenous insulin could not be demonstrated in their diabetic rats, indicating that increased shunting in response to insulin administration does not occur in diabetic rats and consequently can not account for the observed laser Doppler flux increments. Moreover, as in our study, Kihara's study did not differentiate between the effects of insulin and glucose on the nerve vasculature; insulin was infused together with glucose and the blood glucose values in their non-diabetic rats were approximately 15 mmol l<sup>-1</sup>. Since we infused equal amounts of insulin in all diabetic rats and there was correlation between plasma glucose reduction and the change in nerve Doppler flux, we suggest that plasma glucose concentration may be the prime

determinant. Future studies in which insulin is infused close-arterially while plasma glucose is clamped at a fixed level could address this issue. There were no changes in systemic or sciatic nerve function in response to haemaccel infusion in control rats, indicating that the experimental procedure as such did not influence results.

The effect of acute insulin administration on nerve conduction velocity in diabetic animals was not clear. While motor conduction appeared to remain unaffected, sensory conduction velocity was improved. There have been earlier reports of amelioration of nerve conduction deficits within days after insulin administration in streptozotocin-diabetic rats (Julu and Mutamba, 1991) and within hours in diabetic patients (Troni et al., 1984). At present we have no explanation for the differential effects of acute insulin administration on motor and sensory conduction in this study. An acute effect on nerve Doppler flux without a concomitant effect on nerve conduction does not preclude a causal relationship; we have little indication of periodicity and it may be necessary to hold nerve blood flow in the normal range for some time before a robust change in conduction velocity ensues.

In summary, we have shown that insulin is able to reverse an existing deficit in nerve blood flow in streptozotocin-diabetic rats, as determined with laser Doppler flowmetry. The effect of insulin on blood flow occurs after short-term (one month) and acute (one hour) administration. The improvement in blood flow is likely to be related to falls in plasma glucose level, although insulin may also have direct effects on the local vasculature. In association with blood flow improvement, we found an amelioration of nerve conduction velocity but not of the resistance to hypoxic conduction block with short-term reversal insulin treatment. Insulin restored both systemic and local (sciatic nerve) metabolic abnormalities. The data indicate that insulin ameliorates nerve dysfunction in experimental diabetes through glycaemic control and improvement of nerve blood flow.

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