Nerve ischaemia in diabetic rats: time-course of development, effect of insulin treatment plus comparison of streptozotocin and BB models

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Summary This study sought to determine the time-course of development of reduced nerve laser Doppler flux in experimental diabetes and the effect on this anomaly of insulin treatment. In addition, we aimed to compare nerve laser Doppler flux in streptozotocin- and genetically-diabetic BB rat models. Sciatic nerve laser Doppler flux in diabetic rats was variable during the 2 days following streptozotocin injection; from day 4, when the measurement was 80% of control, fluxes fell steadily and formed a plateau at 40% of control values after 4 weeks of diabetes. In a second study, using rats with 4-week streptozotocin-diabetes, sciatic nerve laser Doppler flux was reduced to 44% of the value measured in control rats. Treatment of a parallel group of diabetic rats with insulin, by sustained release implants, prevented this ischaemia, so that nerve laser Doppler flux was 91% of controls. Nerve Doppler flux in BB rats with 6-week genetic diabetes was 57% of a control (non-diabetic) BB group. There were no differences in mean arterial pressures between control and diabetic rats in any of the studies. Heart rates of control and insulin-treated diabetic animals were higher than those of the untreated diabetic group; in the other studies heart rates of diabetic animals were numerically lower than controls, but not significantly so. These observations suggest that sciatic nerves of rats with short-term diabetes, whether induced with streptozotocin or of genetic origin, are markedly ischaemic and that this ischaemia in streptozotocin-diabetes is evident within a week of diabetes onset, forms a plateau after 4 weeks and is maintained for at least 2 months. The findings also indicate that treatment of short-term diabetes with insulin can prevent nerve ischaemia. [Diabetologia (1994) 37: 43–48]

Key words Diabetes mellitus, Doppler flux, ischaemia, rat, insulin, BB rat, streptozotocin.

Reduced nerve conduction velocity, together with exaggerated resistance to anoxia are characteristic of both experimental and clinical diabetes mellitus [1]. The development of these anomalies is associated with both poor blood glucose control and long duration of diabetes [2, 3], as well as genetic and other factors [4]. The aetiology of nerve disorders is still unclear, although current hypotheses offer both biochemical and vascular explanations. The former are concerned with

increased glucose flux through the polyol pathway and, thus, extensive investigation into the potential benefit of blocking aldose reductase, the first enzyme of this pathway, has been pursued. Some inhibitors have prevented conduction abnormalities in experimental diabetes, but clinical effects have been minimal (see [5] for review). Neurovascular insufficiency, leading to nerve ischaemia, has been implicated in the second group of hypotheses. This is supported by findings of reduced endoneurial nutritive flow in experimental diabetes [6, 7] and by low nerve oxygen tension in both diabetic animals [6] and patients [8]. Further support comes from the demonstration that maintenance of persistent central hypoxaemia in non-diabetic rats causes a nerve conduction deficit of similar magnitude to that seen in diabetic rats [9]. Pharmacological inter-

vention aimed at improving nerve blood flow is also, therefore, rational for prevention of nerve functional abnormalities. Indeed, studies with compounds which are likely to attenuate vasoconstriction, such as guanethidine [7], lisinopril [10] and prazosin [11], have prevented slowed conduction in experimental diabetes. Treatment of diabetic rats with evening primrose oil has also attenuated these anomalies [12, 13], as well as preventing diabetes-induced ischaemia [14].

We have previously measured nerve laser Doppler flux (nerve blood flux) as a parameter of nerve blood flow and demonstrated a 50 % reduction in streptozotocin-diabetes [14]. The present study was designed to follow the time-course of development of this abnormality and to determine the effect of treatment with insulin, delivered via sustained release implants for strict glycaemic control, on the diabetes-induced nerve blood flux deficit. We have also compared nerve blood flux in streptozotocin- and BB-diabetic rats.

Materials and methods

Experimental organization

Three separate studies are described; the first two of which used male Wistar rats (290–360 g; 9–13 weeks of age; Charles River (UK) Ltd., Margate, UK) and the third of which used BB (Biobreeding) Wistar rats (Southampton University, Southampton, UK). In the first two studies (a time-course experiment and an insulin study) diabetes was induced with streptozotocin. Rats were fasted overnight and, early the following morning, given a single intraperitoneal injection of 50 mg $\cdot$ kg^{-1} streptozotocin (ICI Pharmaceuticals, Macclesfield, UK) freshly dissolved in sterile saline (0.9 % weight/volume aqueous NaCl). Two days later, blood samples were obtained by tail prick from the streptozotocin-treated rats and blood glucose concentration was measured by strip-operated reflectance photometry (Reflolux, Boehringer Mannheim, Mannheim, Germany). All the animals had blood glucose concentration greater than 15 mmol/l and were included in the studies.

The first study examined the time-course of development of nerve blood flux deficit in streptozotocin-diabetes, using untreated control and diabetic rats for time-points over a total of 8 weeks.

The aim of the second experiment was to determine the effect of treatment with insulin on diabetes-induced changes in nerve blood flux. The control group and one group of diabetic rats were left untreated. Immediately after confirmation of diabetes, in a second group of diabetic rats, two sustained release insulin implants (Linplant; Møllegaard, Ejby, Denmark; release rate 2 IU/day/implant for more than 40 days), were inserted subcutaneously, under halothane anaesthesia, in the back of the neck of each rat. Blood glucose concentration was measured at least weekly and in the second week of the study a 24-h blood glucose profile was monitored in two parts separated by 5 days.

The third study used BB non-diabetic and diabetic rats, with mean (± SD) age at death of 151 ± 41 and 132 ± 31 days, respectively. Diabetic animals had a mean (± SD) duration of diabetes of 45 ± 25 days and had been maintained throughout the protocol on a thrice-weekly insulin regime, using a long-acting heat-treated Ultralente preparation (Novo Industri A/S, Copenhagen, Denmark) [15] designed for minimal glycaemic control.

All rats were weighed at least weekly. Immediately prior to experiment, blood glucose levels were again tested. After death, whole blood and plasma samples were collected for later determination of glycated haemoglobin levels and glucose content, by spectrophotometric assay (GOD-PERID test; Boehringer Mannheim), respectively.

Sciatic nerve Doppler flux

Animals were anaesthetised initially with halothane (4 % in O_2 for induction, 2–2.5 % for maintenance) and a left jugular venous catheter inserted for infusion of alphaxalone 9 mg/ml and alphadolone acetate 3 mg/ml (Saffan; Pitman-Moore Ltd, Uxbridge, UK). The manufacturer's solution was diluted 1:3 with 0.9 % NaCl and an initial dose of 4 mg/kg body weight of this dilution was given on removal of the rats from the halothane source; the solution was infused slowly as the halothane was breathed off. Anaesthesia was maintained by continuous infusion of a 1:6 solution of alphaxalone/alphadolone at a rate of 12 mg/h. The left carotid artery was cannulated (PVC Intravenous Cannula – outer diameter 1.34 mm, inner diameter 1.00 mm; Portex, Hythe, UK) and connected to a transducer (Type 4-327 L221; Bell & Howell, Basingstoke, UK) for measurement of systemic arterial pressure and heart rate. Via a small incision in the left flank the sciatic nerve was exposed. A fibre optic flow probe (Type P4; tip diameter 0.85 mm; Moor Instruments, Axminster, UK) was manoeuvred using a X-Y-Z micropositioner (Prior Scientific Instruments, Bishop's Stortford, UK) until it was just in contact with the nerve and Doppler flux was registered on the flow monitor (Type MBF3D; Moor Instruments). Measurements were made from each nerve by withdrawal and repositioning of the probe between each. This was repeated until three measurements of similar proportions were obtained (in practice these were usually the first and only three). Both systemic pressure and nerve blood flux outputs were amplified via a MacLab Bridge Amp (AD Instruments Ltd., London, UK), digitized (MacLab/8 Interface Module; AD Instruments), displayed and recorded simultaneously using MacLab "Chart" software (AD Instruments) with a Macintosh personal computer and monitor (supplied as above). Values for all cardiovascular variables were averaged over a 2-min recording period. Body temperature for all rats was maintained at 37.7 ± 0.5 °C via homeothermic blanket (Harvard Apparatus Ltd., Edenbridge, UK), with biofeedback by rectal probe. Rats were killed without recovery from anaesthesia.

Statistical analysis

Data are presented as mean ± 1 SD. For the insulin study, statistical analyses were carried out by one way analysis of variance (ANOVA). Where the F ratio gave $p < 0.05$, and where there was homogeneity of variance (Cochrans & Bartlett Box, $p > 0.05$), comparisons between individual group means were made using Duncan's Multiple Range Test at significance levels of $p < 0.05$ and $p < 0.01$. Diabetic BB rats were compared with their controls by unpaired t-test.

Results

Animals

In the time-course study, immediately following streptozotocin injection rats lost weight and were hyperglycaemic; the severity of the diabetic state in these ani-

mals progressed with time, as illustrated by the trends shown in Figure 1. Final body weights were (mean ± 1 SD) 467 ± 79 g for control rats and 325 ± 40 g for diabetic rats. At the end of protocols in the other studies, untreated streptozotocin-diabetic rats and diabetic BB rats had characteristically reduced body weights, together with elevated blood glucose, plasma glucose and glycated haemoglobin levels, compared to their respective controls (Table 1). In Study 2, treatment of diabetic animals with insulin prevented these anomalies (Table 1).

Efficacy of insulin treatment

The strict glycaemic control obtained with insulin implants in diabetic animals was demonstrated by the comparison of insulin-treated diabetic rats with controls, showing similar final weights, plasma glucose and glycated haemoglobin levels (Table 1) and by the narrow range of blood glucose values measured throughout a typical 24-h period (Fig. 2).

Sciatic nerve Doppler flux

Study 1. Individual nerve Doppler flux and heart rate values for rats in the time-course study are shown in Figure 1. Nerve fluxes in control animals fell only slightly over the 8-week protocol. In diabetic animals, nerve Doppler flux was variable during the 2 days after streptozotocin injection; however, from day 4, when the flux in diabetic rats was 80% of control, measurements fell steadily and appeared to plateau at 40% of control values after 4 weeks of diabetes. Mean arterial pressures of control and diabetic animals were not different (mean ± 1 SD in mm Hg were 107 ± 11 and 110 ± 9, respectively). From day 4, heart rates of diabetic animals were reduced when com-

pared to those of controls, although, after 8 weeks of diabetes, differences between these groups were not seen.

Study 2. Figure 3 shows individual animal data points with group means ± 1 SD from this study. A 66% reduction in Doppler flux was seen in sciatic nerves of untreated diabetic animals compared to those of controls. Fluxes in nerves of insulin-treated diabetic rats were only marginally lower than those of the control group (91% of control mean and the difference was not significantly different), indicating substantial prevention of the diabetes-associated decrease. There were no differences between group mean arterial pressures
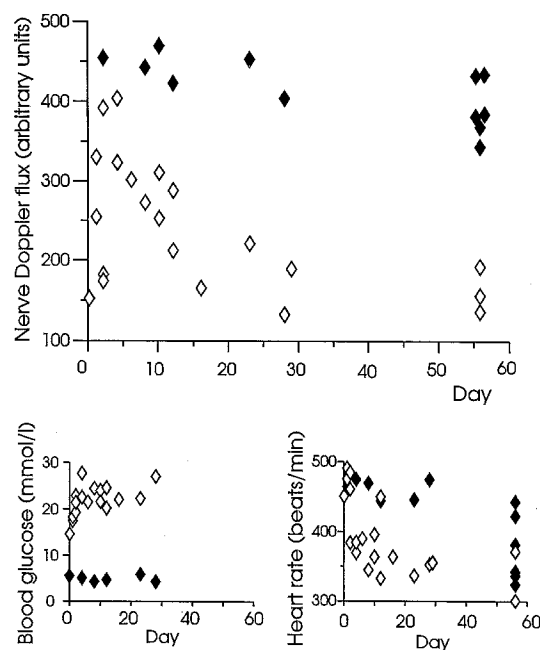

Fig. 1. Time course of development of changes in nerve laser Doppler flux, blood glucose and heart rate in diabetes. Individual points show values for each rat. Control rats, ◆; diabetic rats, ◇

Table 1. Body weight, final plasma glucose, glycated haemoglobin, mean arterial pressure, heart rate and sciatic nerve Doppler flux from the insulin study (Study 2) and from the comparison of control and diabetic BB rats (Study 3)

	Body weight (g)		Plasma glucose (mmol/l)	Glycated haemoglo-bin (%)	Mean arterial pressure (mm Hg)	Heart rate (beats/min)	Nerve Doppler flux (arbitrary units)
	Initial	Final					
Study 2							
Control (10)	321 ± 19	455 ± 31	8.6 ± 0.9	3.7 ± 0.7	114 ± 5.9	426 ± 97	435 ± 53
	NS	$p < 0.01$	$p < 0.01$	$p < 0.01$	NS	NS	$p < 0.01$
Diabetic (9)	321 ± 19	361 ± 38	33.4 ± 6.7	7.5 ± 1.9	112 ± 6.4	355 ± 84	193 ± 62
	NS	$p < 0.05$	$p < 0.01$	$p < 0.01$	NS	$p < 0.05$	$p < 0.01$
Diabetic-insulin (10)	322 ± 17	419 ± 99	8.0 ± 5.5	4.3 ± 0.7	109 ± 13.6	479 ± 128	396 ± 62
Study 3							
Control BB (11)	NM	344 ± 95	8.2 ± 1.6	4.0 ± 1.2	108 ± 11	329 ± 62	336 ± 118
		$p < 0.05$	$p < 0.01$	$p < 0.05$	NS	NS	$p < 0.01$
Diabetic BB (8)	NM	269 ± 39	30.5 ± 4.3	7.3 ± 1.8	106 ± 11	296 ± 40	190 ± 53

Data are mean ± 1 SD; numbers of rats are in parentheses. Data from Study 2 were analysed by one-way analysis of variance with Duncan's multiple range tests. Study 3 data were compared (control vs diabetic) by unpaired t-tests. All p values indicate differences between data immediately above and below. NS, Not significant; NM, not measured
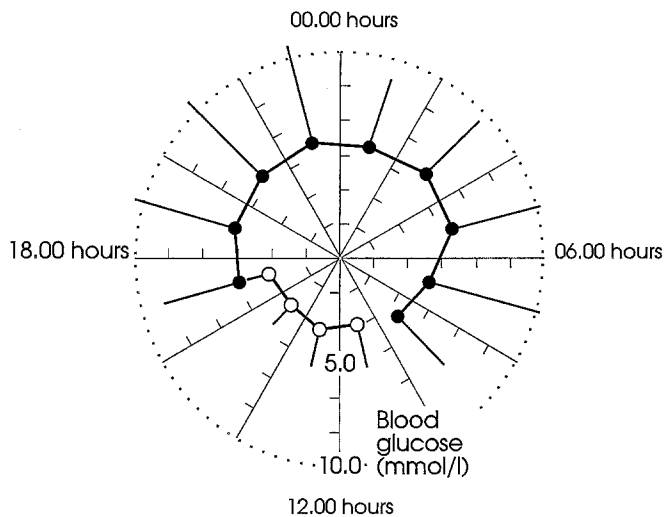
Discussion

Fig. 2. Twenty-four hour profiles of blood glucose in the group of streptozotocin-diabetic rats given sustained insulin delivery implants. ● represent a continuous set of data obtained over one night; ○ represent daytime data obtained 5 days later. Upper limit bars represent calculated standard deviations, though since the data were clearly positively skewed, they are included only to give a rough indication of scatter
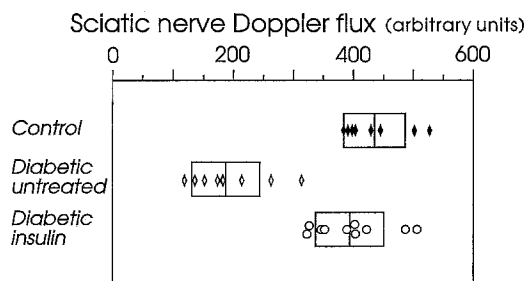

Fig. 3. Effects of diabetes and of treatment with insulin on sciatic nerve laser Doppler flux. Individual points show nerve Doppler flux for each rat, bars indicate group means and boxes show ±1 SD

(Table 1). Heart rates of control and insulin-treated diabetic animals were higher than those of the remaining diabetic groups (Table 1).

Study 3. Group means ±1 SD for nerve Doppler flux, mean arterial pressure and heart rate for BB rats are shown in Table 1. Fluxes for diabetic rats were similar to those found in animals with 2 to 8 weeks of streptozotocin-diabetes and were 57% of their respective control levels. The mean nerve Doppler flux for non-diabetic BB rats was slightly lower than that registered in control Wistar animals. Mean heart rate was again numerically lower in the diabetic group, when compared to controls, but the difference was not significant. There were no differences in group mean arterial pressures.

Untreated streptozotocin-injected rats suffered weight loss, hyperglycaemia and raised levels of glycated haemoglobin, characteristic of experimental Type 1 (insulin-dependent) diabetes. The spontaneously diabetic BB rats also showed reduced body weight and increased levels of both plasma glucose and glycated haemoglobin, when compared to their controls. Insulin delivery reduced the severity of the diabetic state in streptozotocin-treated rats, as indicated by attenuation of the body weight change and normalisation of plasma glucose and glycated haemoglobin levels.

The time-course study indicated that deficits in nerve Doppler flux of streptozotocin-diabetic rats, compared to control animals, are evident within a week following induction of the disease, progress over the next 3 weeks, form a plateau at about 40% of control values after 4 weeks and are sustained at this level for at least another 4 weeks. Cameron et al. [7] also found early changes in sciatic nerve blood flow, albeit endoneurial flow, of diabetic rats, as measured by hydrogen clearance. After 1 week of diabetes, blood flow reductions were slightly larger (59% of control values) than those found in the present study, but remained at this level for 4 months. The hydrogen clearance technique may underestimate reductions in flow, due to diffusion of gas from the nerve during measurement [7], which would account for the relatively larger changes in streptozotocin-diabetes seen at later time-points in the present study. Data from both the time-course and insulin studies, after 4 and 8 weeks of diabetes, show marked reductions in fluxes of untreated diabetic animals, compared to controls; this is consistent with a mean flux of 47% of the control mean, in 5-week diabetic animals found in a previous study [14]. Similar reductions after 1 or more months of streptozotocin-diabetes in rats have also been found using laser Doppler velocimetry [16] and other techniques for measuring both endoneurial and whole nerve blood flow [6, 17–20], although, as mentioned, those determined by hydrogen clearance may underestimate differences. One group failed to find reduced nerve blood flow in diabetic animals, using hydrogen clearance [21], although an explanation for this is unclear. It should also be noted that, in contrast to other studies, Corbett et al [22] and Sutera et al. [23], using radiolabelled microspheres to determine nerve perfusion, consistently found increases in flow in nerves of diabetic rats, compared to controls. This may reflect accumulation of label in vessels, normally of greater diameter than the microspheres, because of vascular compression due to the reported expansion of endoneurial space in experimental diabetes [24].

The limitations of laser Doppler flux measurements as parameters of nerve blood flow were discussed in detail in our previous study [14], but some further consideration should be given here. Nerve Doppler flux readings are not direct measures of blood flow but are

dependent on the number and velocity of erythrocytes passing the probe and, therefore, give an index of whole blood flow. This method does not, therefore, give absolute flow values, nor can flow between nerve compartments be distinguished. Other studies have, however, demonstrated linear correlations of this method with hydrogen clearance [25] and iodo-[^{14}C]antipyrine [26] methods for measuring nerve blood flow at steady state. Laser Doppler velocimetry is therefore recommended for relative estimations of nerve blood flow [25], especially in disease states [26]. We suggest, therefore, that observed reductions in Doppler flux in nerves of diabetic animals, compared to those of controls, are indicative of reduced sciatic nerve blood flow. We cannot discriminate between epineurial and endoneurial vessels, but, as endoneurial flow accounts for approximately 50 % of whole nerve flow [26] and is primarily delivered via epineurial vessels [27], reductions approaching 60 % in short-term diabetic rats, relative to controls, are unlikely to be due solely to lowered epineurial flow. It is, therefore, logical to suggest that the data reflect deficits in sciatic nerve blood flow in streptozotocin-diabetic animals and that decreases in endoneurial nutritive flow may have contributed. The magnitude of changes in nerve flux in experimental diabetes are, thus, likely to indicate debilitating ischaemia. This is supported by the exaggerated resistance to ischaemic conduction block and reduced nerve oxygen tension of diabetic animals [6, 28] and may contribute to their slowed conduction [1, 29]. It is possible that nerve laser Doppler velocimetry overestimates diabetes-associated deficits in flow because of its dependence on movement of erythrocytes, which are poorly deformable in this disease [30].

Nerve fluxes of control animals fell only slightly over the 8 weeks of the time-course study, although body weight progressively increased (data not shown). Such a decline was not evident in another study, which used older animals for an extended protocol period [7]. It is, thus, unlikely that Doppler flux is related to body weight, which may have artifactually resulted in deficient nerve fluxes of the smaller diabetic animals. Of interest, Kihara et al. [31] have reported that nerve blood flow declines with increasing age due to reduced microvascular calibre, although this effect occurred over a longer time-course than that of the control animals used in this study.

Tight glycaemic control of streptozotocin-diabetic rats with insulin implants prevented the diabetes-induced deficit in nerve Doppler flux and we suggest that this was due to amelioration of the diabetic state. We should state that the respective roles of prevention of hyperglycaemia or of hypoinsulinaemia per se by insulin treatment are not defined. Cameron et al. [7] have reported reductions in nerve blood flow of rats made hyperglycaemic by glucose infusion which were similar to those of streptozotocin-diabetic animals, indicating hyperglycaemia as a primary cause.

Our final study reported decreased nerve fluxes in 6-week diabetic BB rats, relative to their controls, which was of slightly increased proportions compared to the changes seen in 4-week streptozotocin-diabetes. This indicates the presence of peripheral nerve ischaemia in this model of Type 1 diabetes, albeit of a lesser degree than that found in acute streptozotocin-diabetes. Although, to our knowledge, nerve blood flow of spontaneously diabetic BB rats has not previously been measured, the finding is consistent with reported nerve conduction abnormalities of these animals, similar to those of streptozotocin-diabetic rats [32].

Systemic arterial pressures did not vary between groups suggesting that changes in sciatic nerve Doppler flux were due to local vasomotor anomalies in diabetes. These could include altered release of cyclooxygenase products and nitric oxide – phenomena which have both been demonstrated in experimental diabetes [33, 34]. Heart rates of control and insulin-treated diabetic animals were consistently higher than those of respective groups of diabetic rats. Bradycardia of streptozotocin-diabetic rats has repeatedly been reported and may reflect disturbances of cardiac autonomic innervation [35, 36] or altered pacemaker potentials due, perhaps, to deficient Na^+/K^+-ATPase activity [37, 38]. However, as arterial pressures in diabetic animals did not differ from control values, it is unlikely that the diabetes-induced reductions in peripheral nerve blood flux resulted from a general vascular insufficiency.

In summary, we suggest that reduced nerve Doppler flux seen in short-term diabetic animals reflects defective nerve blood supply, indicative of an ischaemia, which occurs in both the streptozotocin- and spontaneously-diabetic (BB) rat. In streptozotocin diabetes, neurovascular insufficiency presents within a week of onset of the disease, forms a plateau after 4 weeks and is maintained for at least 2 months. The data indicate that intensive treatment with insulin can prevent nerve ischaemia.

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