Local human sural nerve blood flow in diabetic and other polyneuropathies

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Summary
Microangiopathy is considered relevant to the pathogenesis of several forms of peripheral nerve disease, particularly diabetic polyneuropathy. In diabetes, however, it is uncertain whether reductions in mixed nerve trunk blood flow account for early features of polyneuropathy in contrast to later disease, where microvascular changes have been described.

To address this issue, we measured local sural nerve blood flow in patients with mild diabetic polyneuropathy who were enrolled in a clinical trial (n = 26), patients with other polyneuropathies being studied by diagnostic sural nerve biopsy (n = 17), patients with vasculitic polyneuropathy (n = 3) and one patient with rapidly progressive severe diabetic polyneuropathy and lumbosacral plexopathies. Standardized measurements were made at 10 sites along the sural nerve of each patient prior to sural nerve resection for biopsy. We used a laser Doppler flowmetry probe sensitive to red blood cell flux to measure sural nerve blood flow. This was slightly higher in patients with mild diabetes compared with those with other polyneuropathies, but was reduced in patients with vasculitis. In patients with mild diabetes, there was no relationship between sural nerve blood flow and prebiopsy sural nerve action-potential amplitude, sural myelinated fibre density, haemoglobin A1C, duration of diabetes or age of the patient. Ten diabetic patients entered the clinical trial had sural nerve blood flow recorded in one sural nerve, followed 1 year later by a second sural nerve blood flow measurement prior to biopsy of the contralateral sural nerve. Despite a mild trend toward decline in fibre density between the nerves over this period of time, sural nerve blood flow was similar. The patient with severe diabetic polyneuropathy and lumbosacral plexopathies had reduced sural nerve blood flow. Our findings do not provide evidence that reductions in sural nerve blood flow are associated with early peripheral neuropathy in diabetes, unlike vasculitis. The early trend toward slight rises in sural nerve blood flow may be a result of early functional microangiopathy that accompanies nerve dysfunction but does not cause it.

Keywords: ischaemia; vasa nervorum; vasculitis

Introduction
Pathological evidence for microangiopathy in human diabetic polyneuropathy is well described. Several investigators have described the following changes: closed capillaries (Dyck et al., 1985); microvascular thrombosis (Williams et al., 1980; Timperley et al., 1985); basement membrane thickening (Malik et al., 1989; Giannini and Dyck 1994); endothelial cell reduplication (Williams et al., 1980; Timperley et al., 1985; Giannini and Dyck 1994); and multifocal loss of myelinated fibres (Dyck et al., 1986a, b; Johnson et al., 1986). Despite this evidence, it is controversial whether microangiopathy is the cause of early human diabetic polyneuropathy because these changes occur in already established neuropathy. This problem has been addressed, but not resolved in animal models of diabetes. Some investigators, using careful quantitative techniques, have reported reductions in nerve blood flow as early as 1 week following the onset of experimental streptozotocin-induced diabetes in rats (Cameron et al., 1991b). An early fall in local blood flow of experimental diabetes has also been reported by several other groups, but some of these studies have had methodological flaws. These flaws have included using insufficient numbers of laser Doppler flowmetry measurements, lack of temperature control of the preparation, or inappropriately large hydrogen microelectrodes (Yasuda et al., 1989; Hotta et al., 1992, 1995; Kappelle et al., 1993; Stevens et al., 1993, 1994; Stevens and Tomlinson 1995; Wright and Nukada 1994). In other studies investigators have been unable to identify reductions in nerve blood flow in experi-
mental diabetes or have identified increased nerve blood flow (Pugliese et al., 1989; Zochodne and Ho, 1992a, b; Zochodne et al., 1994, 1996a; Tilton et al., 1993, 1995). Technical differences in the preparation and methodology may account for these differences in findings and are reviewed in depth elsewhere (Zochodne, 1996).

In this study, we measured human sural nerve blood flow prior to nerve biopsy in a group of 26 patients being entered in a multicentre clinical trial of mild diabetic polyneuropathy. The measurements were made using a laser Doppler probe sensitive to local red blood cell flux under controlled conditions that have provided, in prior animal work, consistent flux signals with acceptable variability (Takeuchi and Low 1987; Kalichman and Lalonde 1991; Zochodne et al., 1995). The results were compared with sural nerve blood flow measurements in 22 patients who underwent diagnostic sural nerve biopsy that led to a variety of pathological diagnoses and served as ‘disease controls’.

Methods

Patients with mild diabetic polyneuropathy
Twenty-six patients with diabetic polyneuropathy were local participants in a multicentre trial (Alcar multicentre clinical trial; acetyl-t-carnitine; Hoffmann-La Roche). The protocols for the drug trial and for additional sural nerve blood flow measurements prior to biopsy were reviewed and approved by the University of Calgary conjoint ethics committee, and all patients gave informed consent to participate. The protocol required an initial sural nerve biopsy, randomly assigned as right or left, followed 1 year later (after double-blinded therapy) by a contralateral sural nerve biopsy. The patients selected for the trial were required to fulfill modified San Antonio criteria (Consensus Panel, 1988) for the diagnosis of diabetic polyneuropathy (in brief these were the presence in an individual patient of two of four criteria among clinical symptoms of polyneuropathy, clinical signs, reduced vibration perception threshold, abnormal sural or peroneal nerve conduction). Patients had clinically stable diabetes and no evidence of confounding systemic disorders (more detailed inclusion/exclusion criteria for the trial are available from the corresponding author); having a relatively mild disease, with a recordable sural potential of >1.0 µV in amplitude, was one of the inclusion criteria. At the completion of the trial, analysis of results did not demonstrate that acetyl-t-carnitine was of benefit in diabetic polyneuropathy (Hoffmann-La Roche; personal communication).

Patients with other polyneuropathies
Twenty-two patients with various polyneuropathies (Table 1) were studied prior to diagnostic sural nerve biopsy. These patients also gave informed consent for the sural nerve blood flow measurement. Final diagnoses were established using clinical, electrophysiological and pathological criteria. Patients were included in the vasculitis group only if changes of vasculitis were identified in the biopsy.

Sural nerve blood flow
Sural nerve blood flow was measured using a 1.0-mm-diameter fibre-optic probe connected to a laser Doppler flowmeter (Perimed PFD; Smithtown, NY, USA) sensitive to local red blood cell flux. Individual red blood cell flux measurements do not provide quantitative blood flow values in ml/100g/min since the signal is determined by both local red blood cell velocity and the numbers of moving red blood cells from which the reflected laser signal is acquired. To provide quantitative measurements in relative flux units, however, multiple individual signals can be recorded to provide a mean sural nerve blood flow value. Under carefully controlled conditions, this technique provides reproducible data that correlates, in experimental work, with measurements made using other quantitative techniques, such as hydrogen clearance polarography (Takeuchi and Low, 1987; Kalicham and Lalonde, 1991; Zochodne et al., 1995). We used several approaches to control our recording conditions in order to provide consistent quantitative results: (i) measurements were made with the probe just at the epineurial surface of the nerve with care taken not to compress surface vessels or stretch the nerve with excessive retraction; (ii) the probe was routinely positioned at ~90° to the surface of the nerve and it was held in place during the measurement using a micromanipulator; (iii) measurements were made at 10 different sites along the exposed nerve and the final sural nerve blood flow measurement was taken as the mean of these values; (iv) at each site, the probe was left in place until the signal had stabilized before a recording was made; (v) the ambient room lights in the operating theatre were turned off during the recordings; (vi) the same investigator (M.T.) positioned the probe and set up the recordings in all patients.

Recordings were made immediately after the skin over the sural nerve was incised and the nerve was exposed leaving the nerve in its bed, in order to minimize trauma to it. Incisions and later resections of the nerve were made at the level of the lateral malleolus and taken proximally for 6–12 cm. The nerve was not dissected prior to sural nerve blood flow measurements and care was taken to apply local anaesthesia (lidocaine 2.0%) to the skin, but not the epineurial surface of the sural nerve prior to biopsy. Except in one patient described below, epinephrine was not used over the nerve or for skin anaesthesia. Skin and muscle bleeding was controlled by ligation and bipolar cautery, but no nerve vessels had significant bleeding and none required ligation or coagulation.

Electrophysiology, nerve morphometry and statistical analysis
Sural nerve conduction recordings were made using standardized surface stimulating and recording electrodes.
Table 1 Patient details

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Age (years)</th>
<th>Sural nerve potential (µV)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic polyneuropathy</td>
<td>26</td>
<td>52.5 ± 2.0</td>
<td>4.4 ± 0.7</td>
<td>HbA1C = 9.4 ± 0.4. Six IDDM patients, 20 NIDDM patients</td>
</tr>
<tr>
<td>Other polyneuropathies</td>
<td>17</td>
<td>59.6 ± 3.7</td>
<td>1.1 ± 3.7</td>
<td>Diagnosis: cryptogenic (6), CIDP (5), Sjogren’s (1)*, inherited sensitivity to pressure palsy (1), amyloid (1), GBS (1), paraneoplastic (1), monoclonal gammopathy (1)</td>
</tr>
<tr>
<td>Vasculitic neuropathy</td>
<td>3</td>
<td>65.6 ± 1.5</td>
<td>1.3 ± 1.0</td>
<td>Types: severe, confined to PNS (1); mild, confined to PNS, paraneoplastic (1); mild, associated with SLE and Sjogren’s (1)</td>
</tr>
<tr>
<td>Epinephrine treated</td>
<td>1</td>
<td>76</td>
<td></td>
<td>Cryptogenic neuropathy</td>
</tr>
<tr>
<td>Diabetic lumbosacral plexopathy</td>
<td>1</td>
<td>57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Without vasculitis identified in the biopsy. Data are presented as means ± SE. HbA1C = haemoglobin A1C; IDDM = insulin dependent diabetes mellitus; NIDDM = non-insulin dependent diabetes mellitus; CIDP = chronic inflammatory demyelinating polyneuropathy; GBS = Guillain–Barré syndrome; PNS = peripheral nervous system; SLE = systemic lupus erythematosus.

The recording electrode was placed just behind the lateral malleolus and stimulation carried out 120–150 mm proximally. Nerve morphometry was completed at the University of Michigan, under agreement with Hoffmann-LaRoche. Results reported here were released with the formal permission of Hoffmann-LaRoche. Methods were previously reported (Sima et al., 1988).

The data were analysed using means and standard errors, Student’s t tests that were both paired and unpaired (two-tailed; null hypothesis rejected for $P \leq 0.05$) and linear regression analysis.

Results

Patient information is given in Table 1. Sural nerve blood flow in patients with mild diabetic neuropathy measured slightly higher (though not statistically significant) than that in patients with other polyneuropathies, excluding those with vasculitis, epinephrine application or lumbosacral plexopathy (Fig. 1). Mean sural nerve blood flow ($\pm$SE) in patients with mild diabetic polyneuropathy measured 164 ± 5 ($n = 26$) and in patients with other polyneuropathies 149 ± 8 perfusion units ($n = 17$) (diabetics versus other $P = 0.11$). Within the 10 individual red blood cell flux measurements in each diabetic patient used to calculate mean sural nerve blood flow, no subset of low values was identified. Patients with other polyneuropathies had varying diagnoses listed in Table 1. Sural nerve blood flow within these diagnostic categories did not appear to vary with the specific diagnosis. In three patients with vasculitic polyneuropathy, mean sural nerve blood flow was lower (102 ± 12 perfusion units) than values in either mild diabetic polyneuropathy or in other, non-vasculitic polyneuropathies (vasculitis versus other; $P = 0.03$). In one patient with a cryptogenic polyneuropathy, epinephrine (1.0%) was superfused over the epineurium of the sural nerve by the surgeon prior to the blood flow measurement. Sural nerve blood flow in this patient (85 perfusion units) was $>2$ SDs below the mean value in patients with other, non-vasculitic neuropathies, but was comparable with the sural nerve blood flow result in two of the patients with vasculitis (Fig. 1).

One additional diabetic patient, not involved in the clinical trial, underwent a diagnostic sural nerve biopsy because of a rapidly progressive and disabling polyneuropathy. This patient had weight loss, leg pain, distal lower leg weakness, sensory loss to the knees and also evidence of bilateral lumbosacral plexopathy with asymmetric proximal lower limb wasting and weakness. Sural nerve blood flow (104 perfusion units) was $>2$ SD below the mean value of patients...
with mild diabetic polyneuropathy and 1.5 SD below the value of patients with other, non-vasculitic polyneuropathies.

In patients with mild diabetic polyneuropathy, sural nerve blood flow did not correlate with the pre-biopsy sural nerve potential amplitude, sural nerve myelinated fibre density, haemoglobin A1C, duration of diabetes, or age of the patient (Figs 2 and 3). There was a highly significant correlation between sural nerve myelinated fibre density and the prebiopsy sural nerve potential amplitude \( (r = 0.71, \, r^2 = 0.5; \, P < 0.0001) \). There was no correlation between fibre density and sural nerve blood flow in nondiabetic subjects (Fig. 2). Patients with non-insulin dependent diabetes mellitus \((n = 20)\) did not have significantly different sural nerve blood flow from those with insulin dependent diabetes mellitus \((n = 6)\) \((160 \pm 4 \text{ non-insulin dependent diabetes mellitus versus } 176 \pm 13 \text{ insulin dependent diabetes mellitus})\).

In 10 patients, following the 1-year duration of the clinical trial, a repeat sural nerve biopsy was obtained from the contralateral lower limb. Sural nerve blood flow values in the initial biopsied nerve \((160 \pm 6 \text{ perfusion units})\) did not differ significantly from sural nerve blood flow in the second sural nerve \((185 \pm 14 \text{ perfusion units})\) biopsied from the same patient: there was an overall, but not statistically significant trend toward increased blood flow after 1 year. Acetyl-L-carnitine had no influence on these changes. Figure 3 illustrates the changes in sural nerve blood flow, fibre density and sural nerve potential amplitude in 10 patients, who had two measurements, one in each sural nerve 1 year apart. The sural nerve potential amplitude was similar on each side, after 1 year \((3.9 \pm 0.8 \mu\text{V in the first year; } 4.5 \pm 1.0 \mu\text{V in the second year})\) and there was a trend, not statistically significant, toward a decline in myelinated fibre density \((3614 \pm 695/\text{mm}^2 \text{ in the first year; } 3230 \pm 665/\text{mm}^2 \text{ in the second year})\).

**Discussion**

The major findings of this study are as follows. (i) Patients with mild and early diabetic polyneuropathy had local sural...
nerve blood flow that was similar to, if not slightly higher than, sural nerve blood flow in patients with other polyneuropathies; no single patient in this category had sural nerve blood flow that was >2 SDs below the mean value found in patients with other polyneuropathies. (ii) Patients with vasculitis, and a single patient who was treated with epineurial epinephrine prior to resection, had reduced sural nerve blood flow. (iii) Sural nerve blood flow did not correlate with pre-biopsy sural nerve potential amplitude, myelinated fibre density on nerve biopsy, age of the patient, duration of the diabetes or haemoglobin A_1C value. (iv) Over the course of 1 year, sural nerve blood flow did not decline, despite a slight trend toward a lower myelinated fibre density, as compared with the contralateral sural nerve.

Laser Doppler flowmetry may be the only practical and safe technique available for making measurements of sural nerve blood flow in humans. Laser Doppler flowmetry suffers disadvantages of variable individual red blood cell flux values and it does not give blood flow values in ml/100 g/min but its reliability in providing quantitative data has been established in animal studies where additional blood flow measurements using other techniques (not suitable for human work) could be made for comparison. The important caveat to this statement is the requirement for rigorously controlled measurement technique. For example, holding a laser Doppler microprobe by hand can significantly alter red blood cell flux readings, probably because of microtremor. The probe must be rigidly fixed in place at ~90° to the surface of the nerve with a micromanipulator. Ambient room lights also substantially influence the reading and must be turned off. Calculation of blood flow using single or small numbers of readings from the nerve may also increase the variability of the sural nerve blood flow value assigned. All of our recordings were made from an exposed nerve at normal operating-theatre room-temperature. In animal models, nerves may be warmed by bathing them in a mineral oil or saline bath positioned to prevent spillage and can be kept at a constant temperature with a thermost-controlled heating lamp (Zochodne and Ho, 1992a, b, 1994; Zochodne et al., 1994, 1996a). For ethical reasons, an open surgical wound in a patient cannot be filled with bathing solutions; the nerve was kept moist only with small applications of saline. It is not practical or comfortable for patients to be positioned in a way that would mimic the mineral oil nerve ‘pool’ setup used for animal studies. Cold vasoconstriction, in animal work, appears to reduce sural nerve blood flow in diabetes, a change we did not observe (Dines et al., 1995). Since all of our measurements were made at the same temperature and since vasoconstriction from disease was demonstrable in vasculitic neuropathy and epinephrine treated nerve, we believe our conclusions about the lack of influence of early diabetes on sural nerve blood flow are valid.

The laser Doppler flowmetry signal recorded is particularly sensitive to flow in the epineurial circulation directly beneath the probe. It has been argued that increased arteriovenous-shunt flow in the epineurium might distort readings. While there is evidence for increased arteriovenous-shunt flow in some tissues in advanced diabetes, there is no evidence that major distortions in epineurial arteriovenous-shunt flow occurs in early diabetes, as examined in our work. In experimental diabetes, the presence of endoneurial ischaemia resulting in axonal conduction block was easily demonstrable with laser Doppler flowmetry measurement (Zochodne et al., 1996a). Similarly, we did not observe a subset of individual low red blood cell flux readings in diabetics, remote from the influence of arteriovenous shunts.

Finally, it might be argued that ‘disease control’ patients are an unsuitable group with which to compare sural nerve blood flow from diabetes. We would argue the reverse. By studying ‘disease controls’ with fibre loss and no microangiopathy, we eliminated the possibility that fibre loss itself could have reduced sural nerve blood flow in diabetes. There was no correlation between fibre density and sural nerve blood flow in diabetes or nondiabetics, indicating that neither nonspecific fibre loss nor advancement of diabetes complications could have been linked to changes in sural nerve blood flow had we observed them. Patients with ‘other neuropathies’ did not have diagnoses where abnormalities in vasa nervorum are postulated to contribute to disease. Interestingly, a single patient with amyloid neuropathy had sural nerve blood flow comparable to the values of other patients: amyloid has been considered to result in endoneurial ischaemia but our finding provided no support for such a mechanism (Kyle and Dyck, 1993).

Overall, our results do not support the contention that in human diabetic polyneuropathy, pre-existing ischaemia is the inciting factor responsible for the development of clinical and electrophysiological abnormalities. Indeed, the slight rise in sural nerve blood flow compared with other polyneuropathies may be evidence supporting the contention that capillary hyperperfusion precedes later microvascular sclerosis in diabetes (Tooke, 1995). After 1 year of observation, there was a trend toward yet higher measurements of sural nerve blood flow. As discussed above, there is histological evidence of microvascular disease that would be expected to cause ischaemia in later polyneuropathy. Our patient with advanced polyneuropathy and lumbosacral plexopathy with an apparent reduction of sural nerve blood flow was within this category. Ischaemia has been postulated to be responsible for lumbosacral plexopathy since the original serial section study of Raff et al. (1968) of the lumbosacral plexus of a diabetic patient at autopsy. Tesfaye et al. (1993) noted the delayed appearance of fluorescein in the sural nerves of patients with ‘disabling’ diabetes. Fluorescein visualization is not a recognized or quantitative approach to actual flow measurement, but the findings do suggest disease of epi-neurial vessels in later diabetes. Ram et al. (1991) convincingly linked macrovascular atherosclerosis in patients with diabetes to the severity of the neuropathy. Newrick et al. (1986) made direct measurements of endoneurial oxygen tensions in diabetic sural nerves and identified reductions consistent with local
ischaemia. Lowered oxygen tension, however, may not necessarily reflect an overall impairment in local blood flow, but could result from abnormalities in red blood cell rheology (Simpson, 1988) and in transit through selected capillaries. Malik et al. (1992) reported loss of capillaries in mild human diabetic polyneuropathy, but overall vessel lumen area was actually increased, a finding difficult to reconcile with significant ischaemia. The development of diabetic polyneuropathy appears to result from a complex interplay of metabolic and microvascular abnormalities that cause a vicious cycle of fibre loss and ischaemia. Since sural nerve blood flow was not reduced at the time where obvious fibre loss in the sural nerve had occurred, an alternative explanation to local sural nerve ischaemia in the induction of polyneuropathy may be required.

Since our measurements were made only once and in one limited portion of the nerve, ischaemia at another level with downstream axonal degeneration or transient ischaemia with fibre degeneration may have occurred. There could be subtle ischaemia, confined to the endoneurial space, with gradual loss of axons from chronic low grade hypoxia. In human vasculitis, the summated impact of multifocal ischaemia may result in a pattern of relatively symmetrical and predominantly distal polyneuropathy (Zochodne et al., 1996b). In our patients with vasculitis obvious reductions in sural nerve blood flow were observed. In our 26 diabetic patients, however, at least some should have exhibited evidence of ischaemia, if it were an inciting factor. Also, our results do not provide rationale for the use of vasodilators in diabetic neuropathy, an approach that has been advocated experimentally (Cameron et al., 1991a; Robertson et al., 1992; Kappelle et al., 1993) but could worsen postural hypotension from autonomic neuropathy.

Mechanisms other than ischaemia may be more relevant in early diabetes, such as fibre damage from abnormal polyol flux (Sima et al., 1987), abnormalities of antegrade slow axonal transport (Medori et al., 1985) or defects in retrograde transport of neurotrophins. Finally, early fibre loss could be accounted for by disease of the dorsal root ganglion, a mechanism we have been particularly interested in (Zochodne and Ho, 1994; Zochodne, 1996; Zochodne et al., 1994). Features that might render the dorsal root ganglion susceptible to damage in diabetes have been reviewed elsewhere; they include its higher metabolic requirements, lower oxygen tensions, and its deficient blood-tissue barrier (Arvidson 1979; Greene et al., 1979; Kadekaro et al., 1985; Zochodne and Ho 1991).

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