Skin microcirculation in patients with Type I diabetes with and without neuropathy after neurovascular stimulation

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INTRODUCTION

It is widely accepted that macroangiopathy, peripheral neuropathy, infection, haemorheological and metabolic disturbances are important factors in the pathogenesis of tissue breakdown and foot ulceration in diabetic patients. The concept of microvascular dysfunction, however, is still subject to contention. This concept recognizes the fact that numerous secondary, structural and functional disturbances in the microcirculation are present in diabetic patients. Peripheral diabetic neuropathy is associated with microvascular overperfusion under resting conditions and with an impaired hyperaemic response in total skin blood flow [1–5]. Excess arteriovenous shuntflow due to a reduced peripheral sympathetic tone has been reported [6–8]. This pattern of blood flow seems to be controlled by local reflexes whose function may be altered in diabetic neuropathy [1, 9].

Neurogenic inflammation has been shown to be reduced in diabetic patients suffering from peripheral neuropathy [10–13]. Neurovascular malperfusion may be implicated in the development of trophic foot ulceration [12]. Intradermally applied acetylcholine stimulates neurogenic inflammation by an antidromic axon reflex and causes a spreading vasodilatation and a flare response [10, 12, 13]. Acetylcholine also elicits microvascular dilatation by the release of the vasodilators nitric oxide, prostacyclin and the endothelium-derived hyperpolarizing factor from endothelial cells [14–16].

Post-occlusive reactive hyperaemia has been shown to be impaired in patients suffering from peripheral vascular disease with and without diabetes mellitus [17, 18], which may be caused by a low arterial pressure [19]. Otherwise, in diabetic patients without macrovascular disease, total skin blood flow during post-occlusive reactive hyperaemia seems to be unchanged [20–22]. A maldistribution has been shown between nutritional capillary and non-nutritional arteriovenous shuntflow, inde-

Key words: diabetic neuropathy, Type I diabetes, neurogenic inflammation, nutritive capillary blood flow, total skin blood flow.

Abbreviations: CBV, capillary blood cell velocity; LDF, laser Doppler flux.

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ependent of diabetes mellitus, in patients with peripheral vascular disease [18, 22]. Contradictory results for the distribution of nutritional capillary and subapillary microvascular blood flow have been published for diabetic patients without peripheral vascular disease [23, 24].

Accordingly, the purpose of the present study was to investigate total skin microvascular blood flow and nutritional capillary blood flow in diabetic patients with and without evidence of neuropathy after neurovascular simulation with intracutaneously applied acetylcholine.

**METHODS**

Subjects

Two groups of patients with Type I diabetes, one with and one without features of diabetic neuropathy, were investigated and compared with a non-diabetic control group. Full clinical details of the subjects investigated are given in Table 1. All patients were clinically free from peripheral macrovascular disease, as evaluated by palpable foot pulses and segmental blood pressure measurements. Subjects were excluded from the study if they suffered from neuropathy of an origin other than diabetes mellitus, or were on vasoactive drugs or medications known to influence the autonomic nervous system. No patient exhibited a blood glucose level below 4 mmol/l at the start of the study, or suffered from hypoglycaemia before or during the investigation. All subjects refrained from smoking 2 h before the study. The study has been carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and all subjects gave their informed consent.

**Study conditions and investigation procedure**

All measurements were performed in a room with an ambient temperature of 21–24°C. All subjects were allowed to lie down and to acclimatize for a period of about 15 min. The skin microcirculation in the nailfold of the hallux was investigated by laser Doppler flowmetry [laser Doppler flux (LDF)] and videophotometric capillaroscopy [capillary blood cell velocity (CBV)] with the knee in a slightly flexed position. After a steady signal had been obtained, resting LDF and resting CBV were recorded for a period of 3 min. Thereafter, 0.05 ml of acetylcholine (acetycholine chloride 1%; Ciba Vision, Westling, Germany) was injected intradermally just behind the toe nailfold and again the LDF and CBV were recorded at about 2–3 cm away from the injection site. The mean LDF and CBV before and 5 min after stimulation with acetylcholine were calculated. Skin temperature at the dorsum of the hallux was measured before LDF and CBV measurements using an electronic thermometer (Digitmed H11S, Medizin-Messtechnik, Waldkirch, Germany).

**Assessment of neuropathy**

The autonomic nerve function was evaluated by measurement of the heart rate variation with an automated computer-based system (Pro Sci Card II, Linden, Germany) which is described in detail elsewhere [25]. Heart rate variation at rest, spectral analysis of the low-, mid- and high-frequency range, heart rate variation during deep breathing, the Valsalva manoeuvre and the lying-to-standing maximum/minimum ratio were measured and compared with age-related normal ranges [25]. Blood pressure response was tested in the supine position and 1 min after standing. A decrease in systolic pressure of more than 30 mmHg was considered to be pathological. A score of three or more pathological tests was defined as indicative of cardiovascular autonomic neuropathy.

Peripheral somatic neuropathy was assessed by examining the following aspects: the ankle reflexes using a standard tendon reflex hammer, the vibration perception threshold of the great toe by biothesiometry (Vibra Tester 100, PHYWE, Göttingen, Germany) [26], and the thermal perception and heat pain perception thresholds at the dorsum of the foot using a marstock stimulator (Path-Tester, PHYWE).

<table>
<thead>
<tr>
<th>Table 1. Clinical characteristics of the investigated groups. Values are means ± S.E.M. Statistical significance, *P &lt; 0.05.</th>
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</thead>
<tbody>
<tr>
<td><strong>Type I diabetes patients</strong> without neuropathy (n = 10)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
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<tr>
<td>Smoker</td>
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<td>Mean blood glucose at time of investigation (mmol/l)</td>
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<tr>
<td>Duration of diabetes (years)</td>
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<tr>
<td>Haemoglobin A1c (%)</td>
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<td>Plasma creatinine (mg/dl)</td>
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<td>Plasma fibrinogen (mg/dl)</td>
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<tr>
<td>Albuminuria (mg/24 h)</td>
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<tr>
<td>Retinopathy (Yes/No)</td>
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Age-adjusted values in an individual patient greater than the 95th percentile, compared with values from a healthy population, were regarded as abnormal [26].

Diabetic patients were classified as suffering from diabetic neuropathy if the total score of the above-described neurological investigations (including cardiovascular function) exhibited three or more pathological test results.

Assessment of retinopathy

Diabetic retinopathy was assessed using a non-mydriatic fundus camera (Canon CR 4-45-NM, Tokyo, Japan) together with an examination by an ophthalmologist. Retinopathy was graded as present or absent, regardless of the severity of retinal changes.

Blood flow measurements

*Laser Doppler flowmetry.* The technique of laser Doppler flowmetry (MBF 3D, Moor Instruments, Devon, UK) was used to measure total skin microcirculation at the toe nailfold [27, 28]. Laser Doppler flowmetry is a continuous and non-invasive technique of cutaneous blood flow measurement that has been validated using synchronous dynamic capillaroscopy [29]. The capillary component of superficial skin blood flow is physiologically minimal as compared with the subpapillary blood flow. Therefore, the LDF signal is largely indicative of thermoregulatory and subpapillary blood flow [30–32]. Resting LDF (rLDF) and peak LDF (pLDF) were each measured over a period of 3 min. The absolute increase in LDF (ΔLDF) from baseline after axon reflex stimulation was calculated.

*Videophotometric capillaroscopy.* Using capillary microscopy it is possible to study the nutritional capillaries in the skin directly. This is a technique for measuring blood flow that allows a direct estimate of nutritive capillary blood flow alone [31, 32]. Nailfold capillaries of the hallux were displayed on a TV-monitor using a television microscope (Capi Scope, Moritex Europe Ltd, Cambridge, U.K.) at a magnification of 600. The image was stored on videotape for subsequent analysis. One-minute flow was analysed in each of four previous recorded capillaries with a good contrast, clear focus and visible signals for at least 1 min. Mean CBV was calculated from 10 single measurements out of each capillary. CBV was determined using the ‘line shift diagram method’, a computerized videophotometric technique (Cap Image, Dr. Zeintl Software Engineering, Heidelberg, Germany). Using this method a scale is drawn adjacent to the blood vessel and the movement of plasma gaps are displayed in the form of vertical lines in a line shift diagram. The gradient of the lines in the diagram is determined and the velocity is calculated. The advantage of this method is that zero velocities and negative velocities can also be measured and calculated. Resting capillary blood cell velocity (rCBV) and peak capillary blood cell velocity (pCBV) after stimulation with acetylcholine were measured. The absolute increase in CBV (ΔCBV) from baseline after axon reflex stimulation was calculated.

In addition, the perfused capillaries (capillary density) were projected on to a defined area and calculated as number of capillaries per area. The erythrocyte column width was measured at the arterial side of the capillary loop.

Blood tests

Venous blood samples were taken for the determination of HbA1c, creatinine and plasma fibrinogen. HbA1c was only measured in diabetic patients. Urinary albumin was measured in diabetic patients by 24 h urine sampling, using an immuno-diffusion technique.

Statistical analysis

Results are expressed in terms of the arithmetic mean ± S.E.M. The results obtained from measurements of skin blood flow were not normally distributed. Non-parametric methods of statistical analysis have been used. The Mann–Whitney U-test and the Wilcoxon rank sum test were used in the statistical analysis. Multiple regression analysis and the product moment correlation coefficient were used to compare LDF and CBV with skin temperature, age, duration of diabetes, HbA1c and albuminuria.

RESULTS

As shown in Table 1, all groups were comparable with regard to age, sex, creatinine, plasma fibrinogen blood glucose levels or smoking habits. HbA1c was significantly increased in the group of diabetic patients with neuropathy compared with the group without neuropathy. No statistically significant difference was found with regard to duration of diabetes, presence of retinopathy and albuminuria between the diabetic subgroups.

Blood flow measurements

The reproducibility of the laser Doppler flowmetry readings was tested by performing duplicate measurements of neurovascular stimulation on two different days in eight patients. The intraindividual coefficient of variation was 17.5%. LDF was also measured after intradermally injecting 0.05 ml of
0.9% NaCl solution in five control subjects. The mean LDF signal decreased from 97.7 ± 67.4 to 72.5 ± 40.1 after intracutaneous NaCl injection (not significant).

The reproducibility of the CBV measurements was also tested on eight patients. An intraindividual coefficient of variation of 11.0% was found. Again the test procedure was performed using 0.05 ml of NaCl solution instead of acetylcholine on five control subjects. The CBV increased from 165 ± 12 µm/s to 170 ± 13 µm/s after intracutaneous NaCl injection (not significant).

The skin temperatures on the dorsum of the hallux were comparable between the investigated groups (Table 2).

**Total skin microcirculation**

No significant differences were observed in rLDF at baseline between the investigated groups. After neurovascular stimulation there was a strong increase in LDF in the non-diabetic control group (P < 0.005) and in the non-neuropathic diabetic group (P < 0.005), whereas only a slight, albeit significant, increase was observed in the neuropathic diabetic group (P < 0.05). The absolute increase from baseline LDF (∆LDF) was significantly diminished in the neuropathic group compared with the non-neuropathic diabetic group or the non-diabetic control group (Table 2). No relationship was found between total skin microcirculation after axon reflex stimulation and age (r = 0.113; P = 0.27), duration of diabetes (r = 0.068; P = 0.37), HbA1c (r = 0.28; P = 0.07), plasma fibrinogen (r = 0.125; P = 0.26) or albuminuria (r = 0.052; P = 0.42). No significant difference was found in LDF between male and female subjects or between patients with or without retinopathy.

**Capillary blood flow**

No significant difference was found between the investigated groups with respect to capillary density or basal erythrocyte column width. After neurovascular stimulation, erythrocyte column width was significantly higher in the group of diabetic patients without neuropathy compared with the control group (Table 2). After neurovascular stimulation, capillary blood flow increased significantly in the control group and in the group of diabetic patients without neuropathy. In the group of diabetic patients with neuropathy, however, the increase in CBV was not found to be statistically significant. No significant difference between the investigated groups could be observed regarding rCBV, pCBV or ∆CBV (Table 2). A slight association was found between plasma fibrinogen levels and capillary blood flow in response to axon reflex stimulation (r = -0.34, P = 0.03). No relationship between capillary blood flow and age (r = 0.14; P = 0.23), duration of diabetes (r = 0.24; P = 0.11), HbA1c (r = 0.26; P = 0.13), plasma creatinine (r = 0.07; P = 0.36) or albuminuria (r = 0.34; P = 0.09) could be observed. The capillary blood flow response to acetylcholine (∆CBV) was more pronounced in female than in male subjects (76 ± 17 µm/s versus 38 ± 12 µm/s; P < 0.05). The response (∆CBV) was significantly higher in Type I diabetes patients suffering from retinopathy (n = 6) than in patients without retinopathy (91 ± 27 µm/s versus 44 ± 10 µm/s; P < 0.05).

The ratio between rCBV and rLDF, representing the proportion of nutritive capillary blood flow to total skin microcirculation [20], was not significantly different between the investigated groups when comparing the basal readings. A significant decrease in the CBV/LDF ratio was observed after neurovascular stimulation with acetylcholine in the control group and in the diabetic group without neuropathy (P < 0.005 and P < 0.05 respectively). Only an insig-

| Table 2. Microcirculatory measurements in the investigated groups. Values are means ± S.E.M. Statistical significance: *P < 0.05 compared with diabetic patients without neuropathy; †P < 0.05, ††P < 0.01 compared with control group. |
|-------------------------------------------------------|-------------------------------------------------------|-------------------------------------------------------|
| **Type 1 diabetes patients** | **Type 1 diabetes patients** | **Non-diabetic control group (n = 10)** |
| **with neuropathy (n = 10)** | **without neuropathy (n = 10)** |                                          |
| **Toe skin temperature (°C)** | 27.0 ± 1.1 | 28.7 ± 0.9 | 27.9 ± 0.9 |
| **Resting LDF** | 38.5 ± 18.1 | 56.8 ± 26.2 | 37.4 ± 8.0 |
| **Peak LDF** | 55.5 ± 17.2 | 124.5 ± 43.8 | 88.4 ± 19.4 |
| **∆LDF** | 17.5 ± 8.3 | 67.8 ± 19.7 | 51.0 ± 16.2 |
| **Capillary density** | 49.9 ± 3.0 | 53.3 ± 3.8 | 52.6 ± 3.8 |
| (capillaries/cm²) | | | |
| **Resting erythrocyte column width (µm)** | 9.4 ± 1.1 | 11.2 ± 1.1 | 8.6 ± 1.0 |
| **Peak erythrocyte column width (µm)** | 9.7 ± 1.1 | 13.5 ± 1.0† | 9.2 ± 1.0 |
| **Resting CBV (µm/s)** | 201 ± 13 | 241 ± 13 | 227 ± 15 |
| **Peak CBV (µm/s)** | 240 ± 28 | 301 ± 20 | 283 ± 25 |
| **∆CBV (µm/s)** | 43 ± 18 | 61 ± 18 | 56 ± 17 |
significant decrease was found in the diabetic group with neuropathy (Fig. 1).

Conclusions

Diabetes mellitus may lead to a number of neurovascular complications which may be involved in the development of foot ulceration and loss of digits. In recent years it has been postulated that autonomic C-fibre neuropathy and sympathetic denervation of skin vessels may lead to an increased arteriovenous shunt flow and a maldistribution of nutrient capillary blood flow [4, 6, 24, 33]. The neurogenic inflammatory response to injury can also be impaired in diabetes mellitus, which may be an important factor contributing to tissue ulceration and diabetic neuroarthropathy [11, 12]. Neurogenic inflammation is mediated by small nociceptor C-fibres that also subserve pain sensation. An antidromic axon reflex causes a spreading vasodilatation, probably through the action of substance P on microvessels and mast cells [34]. Non-neurogenic total skin blood flow responses appear to be little affected by diabetes in the absence of major-vessel disease because a substantial increase in cutaneous blood flow above resting levels was observed in the following cases: during post-ischaemic hyperaemia [20, 21, 24, 28], vasodilatation during local heating [1], the first part of the Lewis triple response [1, 3, 10] or direct vasodilatation after stimulation with methacholine or sodium nitroprusside [21, 35, 36].

In our investigation intracutaneous injection of acetylcholine was used to stimulate cutaneous blood flow. Acetylcholine elicits vasodilatation through distinct mechanisms; firstly by direct stimulation of the endothelium and the release of vasodilatory substances [13–16]; secondly by the activation of an axon reflex mechanism and the initiation of neurogenic inflammation [10, 12, 13]. In our investigation we have shown that the total vascular response to intradermally applied acetylcholine is significantly impaired in Type I diabetes patients suffering from neuropathic complications but was not affected in patients suffering from microvascular complications such as retinopathy or albuminuria. This finding suggests that the attenuated vascular response in Type I diabetes patients with neuropathy after intracutaneous stimulation with acetylcholine is mainly due to a loss of nociceptive C-fibre function and is less influenced by a disturbed endothelial function. It is also unlikely that the impaired vascular response may be the result of a disturbed integrity of vascular wall since the response was unaffected in diabetic patients without neuropathy. This is in accordance with an unaffected hyperaemic response after ischaemia in diabetic patients without macrovascular disease [20–22] and with an intact endothelium-dependent vasodilatation after stimulation with methacholine in Type I diabetes patients without complications [21]. It could also be conceived that acetylcholine has an endothelium-dependent vasodilatory effect when applied directly into the bloodstream or to the endothelial cells, but mainly acts via a nociceptive axon reflex arc when applied intracutaneously.

In our study the neurovascular vasodilator response could have been induced via two separate pathways. One is via the activation of a neurogenic axon reflex by acetylcholine and the other is through the stimulation of nociceptor fibres by skin trauma [1, 3]. The second pathway seems to have had less influence on our measurements, since intracutaneous injection of NaCl did not significantly alter skin blood flow in our investigation. Acetylcholine stimulates the afferent branch of the axon reflex arc and the flare response can be eliminated by using local anaesthetics and is also absent in denervated skin [10, 13]. It has been shown that acetylcholine has a direct excitatory effect on non-myelinated C-fibres in the saphenous nerve of the cat, which can be eliminated by using hexamethonium but not by atropine [37]. Pilocarpine produces direct vasodilatation identical to that produced by acetylcholine but no flare response [10]. Therefore, the microvascular response of intradermally applied acetylcholine appears to be mediated via the nicotinic action of acetylcholine. In addition, intradermally applied acetylcholine may act via the prejunctional inhibition of the sympathetic neurotransmission by muscarine receptors [38].

Our observations confirm previous investigations showing a diminished increase in total skin blood flow as measured by laser Doppler flowmetry after axon reflex vasodilatation in diabetic patients suffering from diabetic neuropathy [11, 12, 35, 36]. The reduced hyperaemic response in total skin microcirculation to axon reflex stimulation in patients suffering from diabetic neuropathy underlines the need for an intact nerve supply for regulating the thermoregulatory arteriovenous shuntflow. An increased capillary blood pressure, which has been demon-
strated in toe and finger nailfolds of diabetic patients [14, 39, 40], may be a consequence of the opening of arteriovenous shunt vessels. The interaction of sympathetic neuropathy and microvascular blood flow may lead to an increased transmural pressure, thus promoting the formation of neurogenic oedema [33, 41], and may accelerate long-term capillary structural damage [40, 42] and increase vascular leakage [43].

On the other hand, it has been postulated that the sympathetic denervation of precapillary vessels may lead to a maldistribution of capillary blood flow with a striking reduction of nutritive capillary flow. The impaired nutritive capillary blood flow may lead to an impaired exchange of nutrients, oxygen and waste products in the skin. Whereas an increased skin capillary and arteriovenous shunt flow has been observed in diabetic patients under resting conditions [23], a recent investigation demonstrated a significantly impaired nutritional capillary flow in Type I diabetes patients during post-occlusive hyperaemia, indicating a capillary steal phenomenon under ischaemic conditions [24].

In our investigation, videophotometric capillaroscopy revealed patent capillaries and a similar distribution of terminal row capillaries in each of the investigated groups. Our data contradict the hypothesis of advanced morphological microangiopathy in patients suffering from diabetes mellitus. In a recent study, Morris et al. [14] reported a significant increase in finger capillary blood flow after the iontophoresis of acetylcholine, which was accompanied by an increase in capillary pressure in healthy subjects. In accordance with this observation, our data confirm an increase in foot capillary blood flow after intracutaneous injection of acetylcholine. Our study was able to demonstrate in addition that the increase in capillary blood flow is not affected in diabetic patients without neuropathy and is only slightly influenced by diabetic neuropathy.

The ratio between CBV and LDF, representing the distribution of blood flow between total skin microcirculation and nutritive capillary flow, was not significantly different between the investigated groups under basal conditions. After neurovascular stimulation with acetylcholine, a significant decrease in the ratio between nutritive capillary blood flow and total skin blood flow was found in control subjects and diabetic patients without neuropathy but no significant change could be observed in neuropathic diabetic patients. These findings suggest a deterioration in the regulation of precapillary resistance in patients with autonomic C-fibre neuropathy and a maldistribution of skin perfusion with a reduction of total skin blood flow during neurogenic inflammation. Whether this neurovascular malfunction has any effect on the development of trophic tissue ulceration needs further investigation. In contrast to the finding of a decreased nutritive capillary blood flow after post-occlusive ischaemia in diabetic patients [24], our findings do not support a diminished capillary blood flow after neurovascular stimulation with acetylcholine in Type I diabetes patients with or without neuropathy.

In conclusion the present findings support an impaired neurogenic inflammatory response to injury in total skin perfusion in Type I diabetes patients suffering from diabetic neuropathy. In addition the present study demonstrates a maldistribution between total skin blood flow and nutritive capillary blood flow, which is conditioned by a diminished increase in non-nutritional blood flow and is not accompanied by a diminished capillary blood flow in Type I diabetes patients with neuropathy during neurogenic inflammation. There is no evidence for a capillary steal phenomenon during neurovascular stimulation in Type I diabetes patients with or without neuropathy.

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Skin blood flow in diabetic neuropathy


