

Endothelial Dysfunction and the Expression of Endothelial Nitric Oxide Synthetase in Diabetic Neuropathy, Vascular Disease, and Foot Ulceration

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We studied endothelial-mediated microvascular blood flow in neuropathic diabetic patients to determine the association between endothelial regulation of the microcirculation and the expression of endothelial constitutive nitric oxide synthetase (ecNOS) in the skin. Vasodilation on the dorsal foot in response to heating and iontophoresis of acetylcholine (endothelium-dependent) and sodium nitroprusside (endothelium-independent) were measured using single-point laser Doppler and laser Doppler imaging in diabetic patients with neuropathy (DN), with neuropathy and vascular disease (DI), with Charcot arthropathy (DA), and without complications (D), and in healthy control subjects (C). The response to heat was reduced in the DN (321 [21–629] percentage of increase over the baseline, median [interquartile range]) and DI (225 [122–470]) groups but was preserved in the DA (895 [359–1,229]), D (699 [466–1,029]), and C (810 [440–1,064], $P < 0.0001$) groups. The endothelial-mediated response to acetylcholine was reduced in the DN (17 [11–25]), DA (22 [2–34]), and DI (13 [2–30]) groups compared with the D (47 [24–58]) and C (44 [31–70], $P < 0.001$) groups. The non-endothelial-mediated response to sodium nitroprusside was also reduced in the DI (4 [0–18]), DN (17 [9–26]), and DA (21 [11–31]) groups compared with the D (37 [19–41]) and C (44 [26–67], $P < 0.0001$) groups. There was a significant reduction in vasodilation in the DI group compared with all other groups ($P < 0.0001$). Full thickness skin biopsies from the dorsum of the foot of 15 DN, 10 DI, and 11 C study subjects were immunostained with antiserum to human ecNOS, the functional endothelial marker GLUT1, and the anatomical endothelial marker von Willebrand factor. The staining intensity of ecNOS was reduced in both diabetic groups. No differences were found among the three groups in the

staining intensity of von Willebrand factor and GLUT1. We conclude that the endothelium-dependent and endothelium-independent vasodilations are impaired in diabetic patients predisposed to foot ulceration and that neuropathy is the main factor associated with this abnormality. Reduced expression of ecNOS may be a major contributing factor for endothelial dysfunction. These data provide support for a close association of neuropathy and microcirculation in the pathogenesis of foot ulceration. *Diabetes* 47:457–463, 1998

Diabetic foot ulceration and amputation are serious complications of diabetes with considerable morbidity and mortality. The rate of lower limb amputation is 15 times higher in diabetic patients compared with nondiabetic patients, and more than 50% of diabetic amputees need a subsequent amputation of the contralateral limb within 4 years of the loss of the first leg (1). The financial cost to society is also considerable, indicated by the fact that 20% of all hospital admissions among diabetic patients in the U.S. are for foot problems (2). The two main pathogenic factors related to foot problems are diabetic neuropathy and peripheral vascular disease (PVD) (3).

Abnormalities of the microcirculation play a major role in the complications of diabetes. The microcirculation is regulated by neural and humoral factors, and disturbances of the microcirculation may, in turn, play a role in the pathogenesis of diabetic neuropathy. Recently, attention has focused on the important role of the endothelium in the regulation of vascular tone in the micro- and macrovasculature (4–6).

The endothelium secretes both vasodilators, such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor, and vasoconstrictors, such as prostaglandins and endothelin (7). In human diabetes, generalized endothelial dysfunction not only exists in patients with established complications but also may precede and predict their development (8,9).

The aim of the present study was to investigate endothelial-mediated microvascular regulation in diabetic neuropathic patients with and without macrovascular disease. We also sought to determine the association between endothelial regulation of the microcirculation and the expression of endothelial constitutive nitric oxide synthetase (ecNOS) in the skin. We hypothesized that, since patients with neuropathy develop foot ulceration in the absence of macrovascular disease, there would be changes in endothelial-mediated microvascular regulation in neuropathic patients with a his-

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CV, coefficient of variation; ecNOS, endothelial constitutive nitric oxide synthetase; NDS, neuropathy disability score; NO, nitric oxide; NSS, neuropathy symptom score; PVD, peripheral vascular disease; QTS, quantitative sensory testing; TcPO₂, transcutaneous oxygen tension; VPT, vibration perception threshold.

TABLE 1
Characteristics of study subjects

| | Neuropathic (DN) | Charcot arthropathy (DA) | Ischemic-neuropathic (DI) | Diabetic non-neuropathic (D) | Controls (C) |
|--------------------------------------|------------------|--------------------------|---------------------------|------------------------------|--------------|
| <i>n</i> | 33 | 23 | 32 | 13 | 27 |
| Age (years)* | 56 ± 9 | 57 ± 9 | 60 ± 8 | 39 ± 10 | 52 ± 13 |
| M/W | 24/9 | 13/10 | 23/9 | 9/4 | 13/14 |
| Type of diabetes (1/2) | 12/21 | 5/18 | 16/16 | 8/5 | — |
| Diabetes duration (years) | 21 ± 12 | 17 ± 11 | 25 ± 13 | 17 ± 7 | — |
| BMI (kg/m ²) | 30.3 ± 6.8 | 29.5 ± 4.8 | 27.8 ± 4.5 | 26.8 ± 4.5 | 27.5 ± 4.9 |
| HbA _{1c} | 8.7 ± 2.7 | 8.7 ± 2.0 | 8.9 ± 0.9 | 9.9 ± 4.3 | — |
| Albuminuria [†] | | | | | |
| Normo | 19 (59) | 18 (78) | 15 (60) | 12 (92) | 27 (100) |
| Micro | 2 (6) | 1 (4) | 1 (4) | 1 (8) | 0 (0) |
| Macro | 11 (35) | 4 (18) | 9 (36) | 0 (0) | 0 (0) |
| Creatinine (mg/dl) | 1.03 ± 0.3 | 1.01 ± 0.4 | 1.05 ± 0.031 | — | — |
| Retinopathy (%) | 23 (70) | 14 (61) | 19 (65) | 4 (31) | 0 (0) |
| Smokers (no) | 12 (36) | 6 (26) | 16 (59) | 1 (8) | 3 (14) |
| NSS [‡] | 3.2 ± 2.9 | 2.9 ± 2.5 | 3.5 ± 2.8 | 0.5 ± 1.1 | 0.1 ± 0.4 |
| NDS [§] | 19.3 ± 6.1 | 21.8 ± 3.9 | 18.7 ± 6.7 | 0.5 ± 1.2 | 0.4 ± 1.2 |
| VPT (V) | 48 ± 5 | 50 ± 3.4 | 47 ± 8 | 11 ± 5 | 12 ± 6 |
| Semmes-Weinstein monofilaments¶ | 6.6 ± 0.7 | 6.9 ± 0.4 | 6.6 ± 0.5 | 4.0 ± 0.5 | 4.0 ± 0.5 |
| Foot skin temperature (°C)# | 31.7 ± 1.3 | 31.4 ± 1.0 | 30.5 ± 1.3 | 30.1 ± 2.0 | 31.4 ± 1.4 |
| Peroneal motor conduction velocity** | | | | | |
| Measurable | 13 (39.9) | 4 (17) | 9 (28) | 13 (100) | 27 (100) |
| Mean (m/s) | 32.8 ± 7.2 | 28.4 ± 4.2 | 36.3 ± 4.1 | 45.5 ± 5.1 | 48.2 ± 5.2 |
| TcPo ₂ (mmHg)†† | 61 ± 14 | 70 ± 12 | 37 ± 23 | 75 ± 13 | 75 ± 9 |

Data are means ± SD or *n* (%). *D vs. DN, DA, and DI, *P* < 0.01; †DN, DA, and DI vs. D and C, *P* < 0.01; ‡DN, DA, and DI vs. D and C, *P* < 0.001; §DN, DA, and DI vs. D and C, *P* < 0.001; ||DN, DA, and DI vs. D and C, *P* < 0.001; ¶DN, DA, and DI vs. D and C, *P* < 0.001; #DN and DA vs. DI and D, *P* < 0.001; **DN, DA, and DI vs. D and C, *P* < 0.001; ††DI vs. DN, DA, D, and C and DN vs. DA, D, and C, *P* < 0.001.

tory of foot ulceration and that these changes would be associated with reduced cutaneous expression of eNOS.

RESEARCH DESIGN AND METHODS

Patients. All research subjects were recruited from the Deaconess-Joslin Foot Center located at the Beth Israel-Deaconess Medical Center. Five groups of subjects were studied. The first group consisted of 33 diabetic neuropathic patients with a history of foot ulceration but no PVD (DN); the second group of 32 diabetic patients with PVD and neuropathy (DI); the third group of 23 diabetic patients with Charcot arthropathy (DA); the fourth group of 13 diabetic patients without any complications (D); and the fifth group of 27 control subjects without diabetes or any other systemic illness (C). Subjects younger than 18 years and older than 70 years and patients with diabetic nephropathy (creatinine >2 mg/l), severe heart failure, or any other serious illness were excluded from the study.

All participants gave written consent, and the study was approved by the institutional review board. Details of the clinical characteristics of each group are given in Table 1. In brief, the non-neuropathic diabetic patients were younger when compared with the other three diabetic groups, while all groups were matched for sex and the number of smokers (past or current). All four diabetic groups were also matched for the type and duration of diabetes, BMI, HbA_{1c}, creatinine, and the presence of retinopathy. As expected from the selection criteria, all measurements of neuropathy were worse in the first three groups (DN, DI, and DA), while no differences existed between non-neuropathic diabetic patients and control subjects. Finally, the transcutaneous oxygen tension (TcPo₂) was lowest in the ischemic patients and reduced in the neuropathic patients, while no difference existed among patients with Charcot arthropathy, non-neuropathic patients, and control subjects.

Characterization of the neuropathy. Diabetic neuropathy was diagnosed according to the San Antonio Consensus Statement criteria (10). The symptoms were evaluated by using a neuropathy symptom score (NSS) and the clinical signs by using a neuropathy disability score (NDS) (11). Quantitative sensory testing (QTS) included the assessment of vibration perception threshold (VPT) using a Biothesiometer and cutaneous perception threshold using Semmes-Weinstein

monofilaments (12,13). The peroneal motor nerve conduction velocity was measured using surface electrodes (14). The diagnosis of Charcot neuroarthropathy was made when gross destruction of the joints of the mid-foot that resulted in significant foot deformity was present.

Evaluation of ischemia/hypoxia. Patients were characterized as having PVD based on the presence of one or more of the following clinical features: claudication, absent foot pulses, and/or abnormal invasive and abnormal noninvasive vascular tests. The TcPo₂ measurement at the dorsum of the foot was assessed using a Microspan TcPo₂ meter (BCI International, WI). The electrode was left in place for 20 min, and a stable reading of more than 1 min after this time was used for analysis (15).

Blood flow response to heat. The blood flow response to heat was assessed by using a single-point laser probe and a DRT4 Laser Doppler Blood Flow Monitor (Moor Instruments, Millwey, Devon, England). The blood flow was measured at the dorsum of the foot while the patient rested in a warm environment (room temperature 23–24°C). After baseline measurements were recorded, the skin was heated to 44°C for 20 min using a small brass heater (Moor Instruments), and the maximum blood flow was then remeasured. The results were expressed in arbitrary units of flux.

Laser Doppler iontophoresis. Endothelial-mediated vasodilation was measured by the iontophoresis of acetylcholine, while sodium nitroprusside was used to measure endothelium-independent vasodilation. The iontophoresis instrument (MIC1 iontophoresis system [Moor Instruments]) consists of an iontophoresis delivery vehicle device that sticks firmly to the skin with the help of adhesive tape. The device contains two chambers that accommodate two single-point laser probes. One probe is placed within the chamber containing the iontophoresis solution (thus measuring the direct response to acetylcholine iontophoresis), while the second probe is placed outside but in close proximity to (5 mm) the iontophoresis solution chamber (thus measuring the indirect response). The indirect vasodilatory response is due to stimulation of the C nociceptor fibers and therefore measures the axon reflex-mediated vasodilation. A small quantity (<1 ml) of 1% acetylcholine chloride solution or 1% of sodium nitroprusside was applied to the iontophoresis chamber at the dorsum of the patient's foot. Subsequently, a constant current of 200 µA for 60 s was applied, achieving a dose of 6 mC · cm⁻²

TABLE 2
Iontophoresis results

| | Neuropathic (DN) | Charcot arthropathy (DA) | Ischemic- neuropathic (DI) | Diabetic non-neuropathic (D) | Controls (C) | <i>P</i> value |
|-----------------------------------|---------------------|--------------------------------|----------------------------------|------------------------------------|---------------------|----------------|
| Single-point laser probe | | | | | | |
| Baseline | 11 (8–15) | 12 (6–15) | 11 (9–17) | 12 (9–17) | 16 (10–22) | NS |
| 44° C | 71 (35–84) | 94 (57–120) | 47 (26–64) | 119 (76–175) | 127 (99–162) | * |
| Percentage increase over baseline | 321 (210–629) | 895 (359–1229) | 225 (122–470) | 699 (466–1029) | 810 (440–1064) | * |
| Acetylcholine | | | | | | |
| Baseline (V) | 0.32 (0.22–0.37) | 0.26 (0.19–0.35) | 0.27 (0.021–0.35) | 0.33 (0.27–0.48) | 0.36 (0.23–0.42) | NS |
| Post-iontophoresis (V) | 0.34 (0.28–0.42) | 0.31 (0.24–0.36) | 0.29 (0.26–0.35) | 0.51 (0.37–0.74) | 0.51 (0.34–0.68) | † |
| Percentage increase over baseline | 17 (11–25) | 22 (2–34) | 13 (2–30) | 47 (24–58) | 44 (31–70) | † |
| Nitroprusside | | | | | | |
| Baseline (V) | 0.33 (0.27–0.42) | 0.32 (0.24–0.34) | 0.30 (0.25–0.42) | 0.34 (0.28–0.47) | 0.32 (0.27–0.39) | NS |
| Post-iontophoresis (V) | 0.38 (0.34–0.46) | 0.35 (0.30–0.40) | 0.32 (0.30–0.40) | 0.46 (0.35–0.66) | 0.48 (0.37–0.60) | † |
| Percentage increase over baseline | 17 (9–26) | 21 (11–31) | 4 (0–18) | 37 (19–41) | 44 (26–67) | ‡ |

Data are median (interquartile range). **P* < 0.0001, DN and DI vs. DA, D, and C; †*P* < 0.0001, DN, DA, and DI vs. D and C; ‡*P* < 0.0001, DI vs. DN and DA vs. D and C.

between the iontophoresis chamber and a second nonactive electrode placed 10–15 cm proximally to the chamber. This current caused a movement of the iontophorized substance toward the skin and resulted in vasodilation, which was recorded by the two laser probes.

We also measured the response to the iontophoresis of acetylcholine and sodium nitroprusside using a laser Doppler perfusion imager (Lisca PIM 1.0, Lisca Development, Linköping, Sweden) (16). This apparatus employs a 1 mW helium-neon laser beam of 633 nm wavelength that sequentially scans an area of the foot. The maximum number of measured spots is 4,096, and the apparatus produces a color-coded image of skin erythrocyte flux on a computer monitor. The scanner was set up to scan 32 × 32 measurement points over an area ~4 × 4 cm.

The day-to-day reproducibility of the technique was evaluated in five healthy subjects (four men and one woman, ages 23–39 years) who were repeatedly tested at their foot and arm for 10 consecutive working days. The coefficient of variation (CV) for the baseline blood flow measurement obtained with the laser probe evaluating the response to heat was 44.0%, while that for the maximal response to heat was 27.9%. The indirect response to acetylcholine, measured by a single-point laser probe, had a CV of 60.6% for the baseline measurements and 35.2% for the maximal hyperemic response after the iontophoresis. The laser scanner had a significantly better reproducibility with a CV before the iontophoresis of acetylcholine of 25.9%; during the maximal response after the iontophoresis of 19.2%; before the iontophoresis of sodium nitroprusside of 14.8%; and after the iontophoresis of 14.3%. At the forearm, the CV before the iontophoresis of acetylcholine was 14.1%, and during maximal hyperemic response after the iontophoresis, it was 13.7%.

Furthermore, in order to prove that the observed differences between the baseline and the post-iontophoresis measurements obtained with the laser scanner were related to the increased blood flow and not to any other nonspecific factor, we performed acetylcholine iontophoresis at the forearm of 26 subjects. Both measurements were performed as described above, but in addition, we scanned the studied area while the blood flow at the forearm was arrested by applying pressure above the systolic pressure through a sphygmomanometer at the subject's arm. The observed measurement during this maneuver, called the biological zero, is related to scattered light. The mean baseline measurement was 0.616 ± 0.066 V (mean ± SD) and the biological zero 0.516 ± 0.053, while the post-iontophoresis measurement was 1.410 ± 0.385 and the post-iontophoresis biological zero 0.512 ± 0.046. The mean difference between baseline and post-iontophoresis biological measurement in these 26 subjects was 0.8%, and the maximal difference in any of the subjects was 8%. These data indicate that the observed dif-

ferences before and after iontophoresis were related to changes in blood flow.

Tissue specimens. Skin biopsies, taken from the dorsum of the foot, were snap-frozen using 2-methylbutane cooled in liquid nitrogen, and the tissue was maintained in the frozen state. The cryopreserved tissue was subsequently submitted for cryostat sections and fixed in cold acetone. The tissue was subjected to immunohistochemical stains for eNOS (both polyclonal and monoclonal), the functional endothelial marker GLUT1, and endothelial cell marker factor III (von Willebrand factor). Immunohistochemical reactions were evaluated independently by two of us (J.P. and U.D.) who were blinded as to the medical history of the patients involved in the study. A semiquantitative scale was used to rate the overall staining intensity in accordance with previously described techniques (17,18). In brief, the scale was as follows: 2+, normal staining; 1+, diminished staining; 0, feeble (>90% reduction). The results were recorded and tabulated before revealing the patient category assignments.

Data analysis. The Minitab statistical package (State College, PA) for personal computers was used for the statistical analysis. For parametrically distributed data, the analysis of variance test was used, followed by the Fisher test to identify differences among the various groups. For nonparametrically distributed data, the Kruskal-Wallis test was used. Finally, the Spearman correlation coefficient *r* was used for the correlation of different parameters in the same group.

RESULTS

The results of the iontophoresis are shown in Table 2. No difference was found at the baseline blood flow measurements obtained either by the single laser probe or the laser scanner imager. The maximum laser flux after heating the skin to 44°C was reduced in the neuropathic and ischemic patients (DN and DI), while no difference existed among the remaining three groups. Similar results were found when the percentage of increase over the baseline flux was calculated (Fig. 1A).

The response to the iontophoresis of acetylcholine, measured with the laser scanner, was reduced in the patients with neuropathy, vascular disease, and arthropathy, while no difference was found between nonneuropathic patients and control subjects. The response to the iontophoresis of

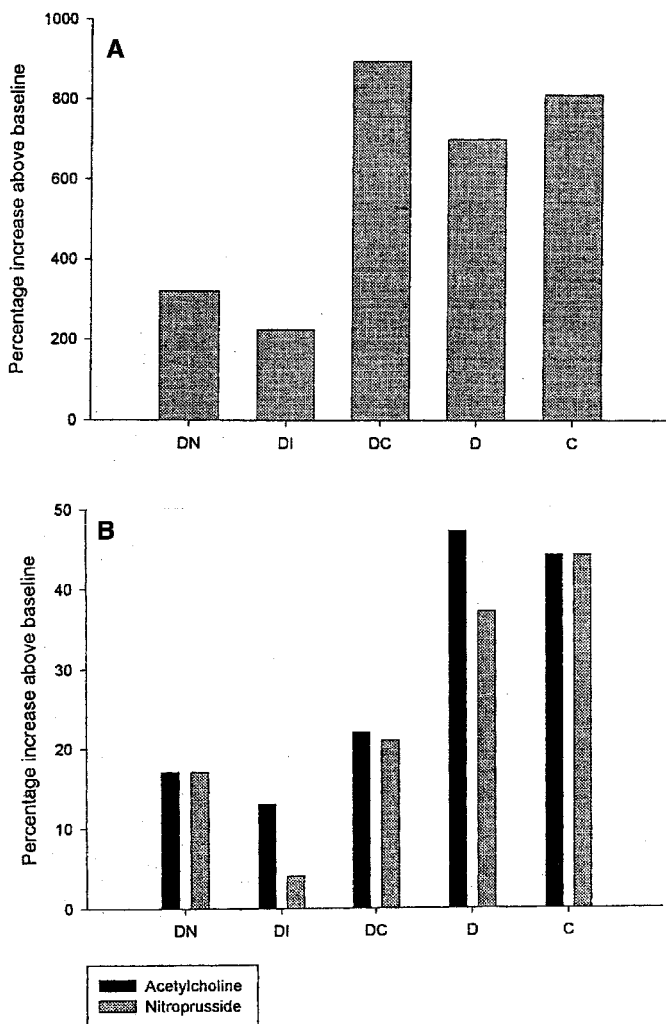


FIG. 1. A: The response to heating the foot skin at 44°C for at least 20 min (expressed as the percentage of increase over baseline flow and measured by a single-point laser probe) was reduced in the DN and DI groups when compared with the DA, D, and C groups, $P < 0.0001$. **B:** The response to the iontophoresis of acetylcholine and sodium nitroprusside expressed as a percentage of increase over baseline flow and measured by a laser scanner imager. The response to acetylcholine was equally reduced in the DN, DI, and DA groups when compared with the D and C groups, $P < 0.0001$. The response to sodium nitroprusside was more pronounced in the DI group and also reduced in the DN and DA groups compared with the D and C groups, $P < 0.0001$.

sodium nitroprusside was more severely reduced in the DI group compared with other groups and reduced in the DN and DA groups compared with the D and C groups. The same results were obtained when the maximum response or the percentage increase over baseline was entered for analysis (Fig. 1B). Similar results were obtained when a single laser probe was used to measure the response to iontophoresis at a skin area that was in direct contact with the iontophoresis fluid (data not shown).

To evaluate the degree of vasodilation that is not specific to the iontophorized substance, we used the single-point laser probe to measure blood flow in the skin area in direct contact with the iontophoresis fluid. First, we measured the vasodilation produced by the iontophoresis of deionized water, which

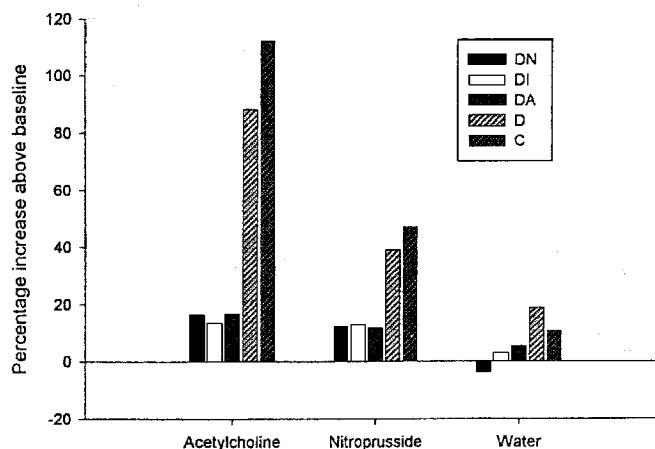


FIG. 2. The response of blood flow (percentage of increase over baseline, measured by a single-point laser probe) in a skin area adjacent to, but not in direct contact with, the iontophoresis solution. During the iontophoresis of deionized water, a mild response was observed in all groups. In contrast, during the iontophoresis of acetylcholine, the response was reduced in the DN, DI, and DA groups when compared with the D and C groups, $P < 0.0001$. A similar response was observed during the iontophoresis of sodium nitroprusside, but it was less than half when compared with the response achieved with acetylcholine.

is the vehicle we used for both the acetylcholine and sodium nitroprusside solutions, and then that produced by the active substance. For the whole group of tested subjects, the median vasodilation during the iontophoresis of deionized water was only 12.3% of the vasodilation achieved by the iontophoresis of acetylcholine (DN 7.3%, DA 8.4%, DI 21.7%, D 25.3%, and C 13%). Similar results were obtained with sodium nitroprusside (median vasodilation for all subjects considered together 11%, DN 7.9%, DA 16.0%, DI 16.5%, D 12.1%, and C 7.6%). These data indicate that most of the observed vasodilation was specifically related to the iontophorized substances.

The indirect or axon reflex response is shown in Fig. 2. A small response was observed during the iontophoresis of deionized water in the nonneuropathic patients and the control subjects compared with the neuropathic groups (DN -3.2 [-13.0 to 11.6] [median, 1st and 3rd quartiles, percentage of increase over baseline], DA 5.4 [-0.6 to 11], DI 3.0 [6.1-9.3], D 18.8 [6.9-22.0], and C 10.7 [1.3-20.0], $P < 0.01$). The response to the iontophoresis of acetylcholine was also reduced in the three neuropathic groups when compared with the last two groups (DN 16.2 [1.6-45.3], DA 16.6 [5.4-63.3], DI 13.5 [-2.2 to 48.5], D 88.2 [27.9-349.8], and C 112.1 [44.3-239.6], $P < 0.0001$). Finally, the response to the iontophoresis of sodium nitroprusside, a solution that contains anions but does not specifically stimulate C nociceptive fibers, was still higher in the two nonneuropathic groups but was less than half of the response obtained with acetylcholine (DN 12.1 [-2.9 to 22.1], DA 11.7 [-1.1 to 22.4], DI 12.9 [1.1-18.1], D 38.9 [14.6-39.4], and C 47 [19.7-91.9], $P < 0.0001$).

No differences in any of the above measurements were observed between men and women when all participants were analyzed as one group or when each group was analyzed separately.

Results of skin biopsies. Thirty-six subjects had skin biopsies taken from the dorsum of the foot. As shown in Table 3

TABLE 3
Characteristics of subjects who underwent skin biopsies

| | Neuropathic (DN) | Ischemic-neuropathic (DI) | Controls (C) | P value |
|---|------------------|---------------------------|--------------|---------|
| <i>n</i> | 15 | 10 | 11 | NS |
| Age (years) | 57 ± 8 | 63 ± 9 | 54 ± 15 | NS |
| M/W | 12/3 | 7/3 | 6/5 | NS |
| Type of diabetes (1/2) | 6/9 | 2/8 | — | NS |
| Diabetes duration (years) | 21 ± 11 | 19 ± 12 | — | NS |
| BMI (kg/m ²) | 28.4 ± 6.4 | 27.8 ± 5.7 | 30.0 ± 3.6 | NS |
| Creatinine (mg/dl) | 1.0 ± 0.2 | 1.00 ± 0.3 | — | NS |
| Retinopathy | 12 (80) | 7 (70) | — | NS |
| NSS | 4 ± 4 | 3 ± 1 | 0 ± 0 | * |
| NDS | 20 ± 6 | 20 ± 4 | 1 ± 1 | † |
| VPT (V) | 48 ± 6 | 50 ± 1 | 14 ± 8 | † |
| Semmes-Weinstein monofilaments | 6.7 ± 0.6 | 6.5 ± 0.7 | 4.0 ± 0.6 | † |
| Measurable peroneal motor conduction velocity | 3 (20) | 2 (20) | 10 (100) | — |
| TcPO ₂ (mmHg) | 61 ± 13 | 50 ± 16 | 76 ± 11 | ‡ |

Data are means ± SD or *n* (%). **P* < 0.05, DN and DI vs. C; †*P* < 0.001, DN and DI vs. C; ‡*P* < 0.01, DI vs. DN and C.

there were no major differences in the subject characteristics and microcirculation functional measurements (including iontophoresis results) of the subgroup that underwent skin biopsies and the whole group. There were also no differences observed between men and women in any of the three groups.

The results of the staining for each factor are shown in Table 4. In summary, staining for von Willebrand factor was present in all studied biopsies and was graded as reduced or present in a similar proportion of subjects in all three groups. A similar proportion of subjects also had absent, reduced, or normal staining for GLUT1. In contrast, the staining for eNOS was reduced or absent in a higher percentage of patients from the two diabetic groups when compared with the control subjects. For technical reasons, staining for von Willebrand factor was not obtained in one biopsy and for GLUT1 in three biopsies.

DISCUSSION

In the present study we have shown that diabetic neuropathy is associated with microcirculation impairment in the form of reduced endothelium-dependent and endothelium-independent vasodilations at the foot level even in the absence of large vessel PVD. We have also shown that there is reduced expression of eNOS in diabetic neuropathic patients regardless of the presence or absence of macrovascular disease. These results suggest that this interaction between neuropathy and endothelial dysfunction results in an inability to increase the blood flow in the diabetic foot under conditions of stress, permitting the development of foot ulceration.

Early studies of diabetic patients who underwent amputation led to the misconception of so-called small vessel disease, which was thought to represent excessive arteriolar occlusive disease (19). Although subsequent controlled studies disproved such pathological findings, the introduction of techniques to measure skin blood flow showed that diabetic patients had reduced maximal hyperemic response to heat even in the early stages of the disease, an abnormality that was believed to indicate that functional microvascular impairment is a major contributing factor for the development of diabetic foot problems (20–23). Our results confirm such func-

tional changes and also show impaired endothelium-dependent and endothelium-independent vasodilations in neuropathic patients with or without PVD. They also imply that the main reason for this reduced microvascular reserve was the presence of neuropathy, as indicated by the fact that no abnormalities were found in nonneuropathic diabetic patients. Further support for this claim is provided by the finding that the coexistence of neuropathy and PVD did not result in a greater decrease in endothelium-dependent vasodilation than that due to neuropathy alone.

In healthy subjects, the ability to increase blood flow depends on the existence of a normal neurogenic vascular response. More specifically, stimulation of the C nociceptive fibers leads to antidromic stimulation of adjacent C fibers, which secrete vasodilators and cause increased local blood flow in injured tissues and thereby promote healing. This response is reported to be impaired in diabetic neuropathy

TABLE 4
Results of immunostaining

| | Neuropathic (DN) | Ischemic-neuropathic (DI) | Controls (C) |
|-------------------------------|------------------|---------------------------|--------------|
| von Willebrand factor (VIII) | | | |
| Absent | — | — | — |
| Reduced | 3 (21) | 5 (56) | 4 (36) |
| Normal | 11 (79) | 4 (44) | 7 (64) |
| Glucose transporter 1 (GLUT1) | | | |
| Absent | 3 (20) | 2 (22) | 2 (22) |
| Reduced | 10 (67) | 5 (56) | 5 (56) |
| Normal | 2 (13) | 2 (22) | 2 (22) |
| eNOS* | | | |
| Absent | 1 (7) | 3 (30) | 2 (18) |
| Reduced | 12 (80) | 6 (60) | 3 (27) |
| Normal | 2 (13) | 1 (10) | 6 (55) |

Data are *n* (%). **P* < 0.05.

(24,25). In the present study we have also found a dramatic reduction of the neurogenic vascular response in all three diabetic neuropathic groups. The reduced neurovascular response in conjunction with the observed reduced blood flow during direct iontophoresis of acetylcholine to the skin microvessels in all three neuropathic diabetic groups indicates that both endothelial dysfunction and impaired C fiber function may be responsible for the reduced microvascular reserve.

Initial reports of diabetic neuropathic patients with foot ulceration contained descriptions of warm red feet with easily palpable pulses and distended veins. These findings were attributed to adequate blood flow and increased arteriovenous shunting due to the presence of a coexisting autonomic neuropathy (26–29). Our laser measurements failed to support these findings, as there were no significant changes in the resting blood flow in all studied groups. However, laser Doppler measurements can only evaluate the superficial skin blood flow and cannot detect the existence of deeper arteriovenous shunting. We therefore feel that our results are not in disagreement with those studies that have shown increased saturation of the venous blood and Doppler wave forms that indicate such arteriovenous shunting; simply, this shunting seems to occur in tissues that cannot be reached by the laser beam (27,30,31). The finding in this study that the skin temperature of the neuropathic and Charcot patients was slightly higher is also in agreement with the above observations and further indicates the inability of laser flowmetry to detect arteriovenous shunting.

Another surprising finding was the preservation of the maximal hyperemic response to heat in patients with Charcot neuroarthropathy in conjunction with a normal transcutaneous oxygen tension despite the reduced response to both acetylcholine and sodium nitroprusside. The mechanisms through which heat leads to vasodilation are not clear. Nevertheless, despite this ability to increase the blood flow in certain stimuli, such as heat, the endothelium-dependent and endothelium-independent vasodilations were also reduced in these patients. This finding indicates that neuropathy can be responsible for a functional reduction of the endothelium-dependent and endothelium-independent vasodilations even in the presence of a normal response to other stimuli. Finally, the normal transcutaneous oxygen tension observed in the Charcot patients is also consistent with clinical observations that the development of Charcot neuroarthropathy is extremely rare in the presence of PVD (32).

Compared with previous studies, we have failed to find any significant impairment of the microcirculation in diabetic nonneuropathic patients (16,21,22). We believe that this may be related to several factors. The primary reason may be that, in the present study, a large number of the nonneuropathic subjects had type 1 diabetes of long duration and were therefore less likely to have microvascular complications or to develop such complications in the future. Another less important factor may be that the mean age of this group was lower compared with the three neuropathic diabetic groups. This difference existed because we selected patients with similar diabetes duration, which could be achieved only by including younger patients. We feel that diabetes duration has a stronger influence on complications than does age.

A large number of medications, including ACE inhibitors and lipid-lowering drugs (33,43), have been shown to affect

endothelial function. A large number of our patients with complications were taking one or more of these medications. However, it should be emphasized that these medications have been shown to improve endothelial function, and one would expect that patients not taking such medications would have even worse results. Therefore, treatment of patients with complications with such medications, if anything, potentiates our findings, namely, that endothelium-dependent and endothelium-independent vasodilations are impaired in diabetic patients with complications. Finally, because these medications have a prolonged effect, we do not think that by stopping them for a few hours or days we could claim that our subjects were not taking vasoactive drugs.

NO is the main vasodilator released by the endothelium and causes vasodilation by diffusing into the adjacent vascular smooth muscle cells and stimulating guanylate cyclase to produce cyclic guanosine 3',5'-monophosphate (cGMP), which in turn leads to vasodilation. In the endothelial cell, NO is synthesized from oxidation of L-arginine through the action of the endothelial-specific isoform of NO synthase (ecNOS), which is membrane-associated and constitutively active (35). The ecNOS expression is reduced in the lungs of patients with pulmonary hypertension, a condition that is related to impaired NO production, but there is no information on ecNOS expression in diabetes (17). Our data point to a reduced expression of ecNOS at the foot level of diabetic neuropathic patients, and this may prove to be the main cause of reduced endothelial function. The lack of any significant difference between neuropathic patients with or without vascular disease in conjunction with normal functional measurements in diabetic nonneuropathic patients also suggests an association between reduced expression of the NO synthetase and diabetic neuropathy.

Support for the validity of our immunostaining results is provided by a previous study where no difference was found in the staining of skin biopsies for von Willebrand factor among healthy subjects and type 1 diabetic patients with short (<5 years), intermediate (6–10 years), or long (>10 years) duration of the disease (36). However, the observed equal impairment of the endothelium-independent vasodilation suggests that reduced expression of ecNOS is not the only cause of the inability to increase blood flow. Rather, it indicates that the function of both the endothelium and the smooth muscle cells is impaired. Further studies will be required before reaching a firm conclusion on this subject.

In summary, the present study has shown that the endothelium-dependent and endothelium-independent vasodilations are impaired in diabetic patients predisposed to foot ulceration and that neuropathy is the main factor associated with this abnormality. Reduced expression of ecNOS may be a major contributing factor for the endothelial dysfunction. The data provide support for a close association of neuropathy and microcirculation in the pathogenesis of foot ulceration.

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