Early Endothelial Dysfunction Severely Impairs Skin Blood Flow Response to Local Pressure Application in Streptozotocin-Induced Diabetic Mice

Dominique Sigaud-Roussel, Claire Demiot, Bérengère Fromy, Audrey Koïka, Georges Lefthériotis, Pierre Abraham, and Jean Louis Saumet

Pressure-induced vasodilation (PIV) is a mechanism whereby skin blood flow increases in response to progressive locally applied pressure. Skin blood flow in response to applied pressure decreased severely in diabetic patients as a result of vascular and/or neural impairment. This study was designed to determine the effect of vascular changes on PIV in 1-week streptozotocin-induced diabetic mice. We assessed cutaneous microvascular response to local increasing pressure application measured by laser Doppler flowmetry (LDF) and endothelium-dependent and -independent vasodilation by iontophoretic delivery of acetylcholine and sodium nitroprusside and sciatic motor nerve conduction velocity and morphometry. In control mice, LDF increased 34% from baseline to 0.2 kPa external pressure, showing PIV response. In contrast, diabetic mice had no LDF increase in response to progressive external pressure. Moreover, after iontophoretic delivery of acetylcholine, endothelium-dependent vasodilation was largely attenuated in diabetic mice (25%) compared with control mice (81%), whereas vasodilation to sodium nitroprusside was not different between groups. Nerve function as assessed by sciatic nerve conduction velocity and morphometry did not differ between groups. These findings suggest that endothelial impairment is sufficient to severely alter PIV response, which seems to be highly sensitive to endothelial nitric oxide levels. PIV suppression could favor diabetes complications such as diabetic foot ulcers. *Diabetes* 53:1564–1569, 2004

We recently reported a mechanism that allows skin blood flow to increase in response to progressive locally applied pressure in humans (1) and rats (2). The pressure applied is a nonpainful stimulation, but we showed that pressure-induced vasodilation (PIV) involved nerve C fibers, because it disappeared after chronic treatment with capsaicin in animals and humans (1,2). In addition, Fromy et al. (2) demonstrated a major role for vasodilators such as the calcitonin gene–related peptide and endothelial vasodilators such as nitric oxide (NO) and prostaglandins in animal studies. In a recent study, we observed in diabetic patients an early decrease of skin blood flow in response to locally applied pressure that could be involved in the development of diabetic ulceration (3). However, it was unclear whether skin blood flow alteration during diabetes depends directly on vascular and/or neural alterations.

Diabetes through hyperglycemia is widely known to be a major factor that leads to microvascular and neural complications (4). Indeed, hyperglycemia-induced end-organ damage in diabetes is associated with increased flux of glucose through 1) the polyl metabolic pathway (5,6), 2) accumulation of advanced glycation end products (7,8), 3) increased oxidative stress (9,10), and 4) activation of the protein kinase C pathway (11,12). However, the pathogenesis of the vascular complications of diabetes is controversial (13). Several studies in experimental animal models of diabetes and in human type I and II diabetic patients revealed impaired endothelium-dependent relaxation (14,15). However, many in vitro studies have reported unaltered or enhanced endothelium-dependent relaxation (16,17). In addition to these vascular observations, Coppey et al. (18) reported as early as 1 week of diabetes in rats a sciatic nerve blood flow reduction that preceded the slowing of motor nerve conduction velocity (MNCV). Therefore, it seems that vascular dysfunction may be a major factor in the development of diabetic neuropathy. Indeed, there is evidence that progressive microangiopathy contributes to progressive loss of peripheral and later central neurologic function (19). However, clinical trials using specific inhibitors of various biochemical pathways activated by hyperglycemia have shown modest improvements in neurologic symptoms (20,21). It therefore seems to be of importance to focus on early vascular alteration, because preventing vascular dysfunction could delay the appearance of diabetic neuropathy.

This study was designed to develop a diabetic mouse model that exhibits vascular but not neuropathic changes to study the microvascular response 1) to local pressure increase and 2) to endothelium-dependent and -independent vasodilators. Nerve function was assessed by sciatic nerve conduction velocity and morphometry to verify its integrity. We hypothesized that vascular dysfunction that occurs early in diabetes could be sufficient to alter the PIV response.

© 2004 by the American Diabetes Association.
RESEARCH DESIGN AND METHODS
Male Swiss mice (20–30 g) were kept on a 12/12 h light/dark cycle with food and water available ad libitum. The present investigation was performed in accordance with the guiding principles in the care and use of animals. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, Sigma; in citrate buffer (pH 4.5)) during the fasting state. Control animals received equivalent doses of the citrate buffer solution. Hyperglycemia occurred 2 days after STZ injection and was verified using an Accu-Check Active glucometer (Roche, Lyon, France). We considered mice to be diabetic when blood glucose was ≥16 mmol/l (normal 5–8 mmol/l), and only mice with blood glucose ≥16 mmol/l both on the second day and 1 week after the STZ injection were included in the experimental diabetic group.

The right sciatic nerve was removed and diabetic (n = 10) mice using laser Doppler flowmetry (LDF). This method was described by Fromy et al. (2) using a weightbridge that was adapted to hold a laser Doppler probe at one end (PF415; PeriFux, Perimed, Sweden). The probe was connected to a laser Doppler flowmeter (PF5000 Master; PeriFux).

After general anesthesia, animals were settled in an incubator (MMS, Chelles, France) warmed to 30°C to maintain stable cutaneous temperature. Mice were placed in the prone position, and the head was fixed on a frame followed by a 20-min resting period to stabilize the blood pressure and the cutaneous temperature. Blood pressure was measured using a noninvasive system (ITC, Woodland Hills, CA). The weightbridge was calibrated care-

Study 1: assessment of PIV. Microvascular responses to local pressure in control (n = 10) and diabetic (n = 9) mice were measured by laser Doppler flowmetry (LDF). This was performed 2 days before the experiments to prevent skin irritation during the experiment from confounding the results.

For the experiments, animals were anesthetized by intraperitoneal injection of thiopental sodium (65 mg/kg). The level of anesthesia was determined by testing eye reflexes and tail pinch. At the end of each experiment, animals were killed by an overdose of thiopental.

Study 2: assessment of endothelium-dependent and -independent response. After general anesthesia, animals were settled in an incubator (MMS, Chelles, France) warmed to 30°C to maintain stable cutaneous temperature. Mice were placed in the prone position, and the head was fixed on a frame followed by a 20-min resting period to stabilize the blood pressure and the cutaneous temperature. Blood pressure was measured using a noninvasive system (ITC, Woodland Hills, CA). The weightbridge was calibrated care-

Study 3: assessment of nerve function and structure. MNCV was examined in control (n = 12) and 1-week diabetic (n = 12) mice as proposed by Zochodne et al. (23). MNCV in sciatic-tibial fibers was assessed by stimulating at the exposed sciatic notch and knee while recording the M-wave (compound muscle action potential) from the tibial-innervated dorsal interosseus foot muscles. During recording, the temperature of the site surrounding the nerve was kept constant at 37°C.

Morphometric analysis. The right sciatic nerves were removed and fixed in 4% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) overnight at 4°C, and then washed in 0.1 mol/l phosphate buffer. These fixed samples were postfixed in 2% osmium tetroxide in phosphate buffer, dehydrated through an ascending series of ethanolic concentrations, embedded in Epon, and polymerized. One-micron-thick semithin transverse nerve sections were stained with toluidine blue. Morphometric analysis was performed using a computer-assisted image analysis (Quin, Leica, France) allowing for the determination of myelinated fiber number and size. All counting was duplicated by two different micros-

RESULTS

Body weight and blood glucose and fructosamine levels. One week of diabetes caused a threefold elevation of blood glucose (Table 1). Fructosamine levels were signifi-
cantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-
glycemia. At the time of experimentation, diabetic mice showed increased blood glucose (Table 1). Fructosamine levels were significantly increased twofold (P < 0.001) in diabetic mice compared with control mice, showing long-lasting hyper-

TABLE 1

<table>
<thead>
<tr>
<th>Animals</th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
<th>Fructosamine (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>31 ± 1</td>
<td>161 ± 7</td>
<td>167 ± 9</td>
</tr>
<tr>
<td>Diabetic (n = 9)</td>
<td>22 ± 1*</td>
<td>495 ± 32*</td>
<td>343 ± 23†</td>
</tr>
</tbody>
</table>

*P < 0.01; †P < 0.001 vs. control.

the reduction in LDF is significant at all pressures >0.46 kPa in diabetic mice and for all pressures >1.16 kPa in control mice. *P < 0.05 vs. rest.

FIG. 1. PIV in 1-week diabetic mice (n = 9; ○) and control mice (n = 10; □). The reduction in LDF is significant at all pressures >0.46 kPa in diabetic mice and for all pressures >1.16 kPa in control mice. *P < 0.05 vs. rest.
PIV ALTERATION IN DIABETIC MICE

In the diabetic group, MABP was 87 ± 6 mmHg at rest, 88 ± 7 mmHg at 0.2 kPa, and 87 ± 5 mmHg at the end of the experiment. One-week diabetic mice had a mean resting LDF of 123 ± 11 au and did not show a significant increase in mean LDF at any time during the experiment (Fig. 1). Mean LDF in diabetic mice was not changed at 0.2 kPa (119 ± 9 au) compared with mean resting LDF (0.2 kPa corresponding to the pressure of the maximal value obtained in control mice). With further increases in pressure, mean LDF decreased and was first significantly lower (110 ± 10 au; P < 0.01) than baseline value at 0.46 kPa (Fig. 1).

Endothelium-dependent and -independent responses. We did not observe any current-induced vasodilation during iontophoresis of deionized water with anodal (P = 0.99) and cathodal (P = 0.99) currents in randomly chosen control and diabetic mice.

In the control group, iontophoresis of ACh induced a significant endothelium-dependent vasodilation from 65 ± 16 to 140 ± 33 au (P < 0.01). In the diabetic group, iontophoresis of ACh also produced a significant vasodilation from 80 ± 13 to 98 ± 15 au (P < 0.01). Baseline values did not differ between diabetic and control mice. However, the percentage of endothelium-dependent vasodilation was largely attenuated in diabetic mice (25 ± 4%) compared with control mice (81 ± 25%; P < 0.05; Fig. 2A).

1). A maximal increase in mean LDF occurred at 0.2 kPa (max LDF: 151 ± 15 au; P < 0.05), demonstrating a vasodilation. With further increases in pressure, mean LDF decreased and first became significantly lower (79 ± 11 au; P < 0.05) than baseline value at 1.1 kPa (Fig. 1).

In the diabetic group, MABP was 87 ± 6 mmHg at rest, 88 ± 7 mmHg at 0.2 kPa, and 87 ± 5 mmHg at the end of the experiment. One-week diabetic mice had a mean resting LDF of 123 ± 11 au and did not show a significant increase in mean LDF at any time during the experiment (Fig. 1). Mean LDF in diabetic mice was not changed at 0.2 kPa (119 ± 9 au) compared with mean resting LDF (0.2 kPa corresponding to the pressure of the maximal value obtained in control mice). With further increases in pressure, mean LDF decreased and was first significantly lower (110 ± 10 au; P < 0.01) than baseline value at 0.46 kPa (Fig. 1).

Assessment of nerve function and structure MNCV. After 1 week of diabetes, MNCV showed no difference (47 ± 3 m/s) compared with control mice (48 ± 5 m/s; Fig. 3).

Sciatic nerve morphometric analysis. The morphometric effects of 1-week diabetes in mice are shown in Table 2. There was no difference in total fiber number or in myelinated fiber size between the diabetic and the control groups. Myelinated fiber size distribution was very similar between 1-week diabetic and control mice (Fig. 4).

DISCUSSION

It is generally agreed that diabetic foot development is a consequence of neuropathy. In our study, we reported that PIV that is a natural mechanism protecting the skin from pressure loads was completely abolished at the onset of diabetes. This demonstrates that neuropathy is not essential for the development of pressure-induced ulcerations. That is why it is of great importance to prevent the development of diabetic foot by detecting as soon as possible vascular alterations. To go further, we propose a new physiopathological concept of the cutaneous microcirculatory control by assessing PIV responses.

In the present study, we have demonstrated that PIV is abolished in 1-week diabetic mice that exhibit endothelial dysfunction but no neuropathy. The absence of PIV develop-

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total fiber number</th>
<th>Mean myelinated fiber size (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>3,936 ± 122</td>
<td>29.4 ± 0.2</td>
</tr>
<tr>
<td>Diabetic (n = 8)</td>
<td>3,980 ± 55</td>
<td>29.7 ± 0.2</td>
</tr>
</tbody>
</table>

The control group, MABP was not different between before (92 ± 4 mmHg) and after (93 ± 3 mmHg) ACh delivery. In the diabetic group, MABP was not different between before (97 ± 9 mmHg) and after (98 ± 9 mmHg) ACh delivery.

SNP iontophoresis produced a significant endothelium-independent vasodilation in both diabetic (67 ± 21%) and control (54 ± 15%) mice. There was no significant difference between the diabetic and control groups (Fig. 2B). In the control group, MABP was not different between before (93 ± 3 mmHg) and after (92 ± 3 mmHg) SNP delivery. In the diabetic group, MABP was not different between before (90 ± 5 mmHg) and after (92 ± 8 mmHg) SNP delivery.

the control group, MABP was not different between before (92 ± 4 mmHg) and after (93 ± 3 mmHg) ACh delivery. In the diabetic group, MABP was not different between before (97 ± 9 mmHg) and after (98 ± 9 mmHg) ACh delivery.

SNP iontophoresis produced a significant endothelium-independent vasodilation in both diabetic (67 ± 21%) and control (54 ± 15%) mice. There was no significant difference between the diabetic and control groups (Fig. 2B). In the control group, MABP was not different between before (93 ± 3 mmHg) and after (92 ± 3 mmHg) SNP delivery. In the diabetic group, MABP was not different between before (90 ± 5 mmHg) and after (92 ± 8 mmHg) SNP delivery.

Assessment of nerve function and structure MNCV. After 1 week of diabetes, MNCV showed no difference (47 ± 3 m/s) compared with control mice (48 ± 5 m/s; Fig. 3).

Sciatic nerve morphometric analysis. The morphometric effects of 1-week diabetes in mice are shown in Table 2. There was no difference in total fiber number or in myelinated fiber size between the diabetic and the control groups. Myelinated fiber size distribution was very similar between 1-week diabetic and control mice (Fig. 4).

DISCUSSION

It is generally agreed that diabetic foot development is a consequence of neuropathy. In our study, we reported that PIV that is a natural mechanism protecting the skin from pressure loads was completely abolished at the onset of diabetes. This demonstrates that neuropathy is not essential for the development of pressure-induced ulcerations. That is why it is of great importance to prevent the development of diabetic foot by detecting as soon as possible vascular alterations. To go further, we propose a new physiopathological concept of the cutaneous microcirculatory control by assessing PIV responses.

In the present study, we have demonstrated that PIV is abolished in 1-week diabetic mice that exhibit endothelial dysfunction but no neuropathy. The absence of PIV develop-
opment in diabetic mice suggested that NO-dependent response to external pressure is reduced. In addition to this result, reduction of NO-dependent response to ACh delivery in diabetic mice supports the idea that background levels of NO were reduced at the early stage of diabetes. We proposed that NO level is reduced as early as 1-week of diabetes and that this reduction is significant enough to alter the PIV response in diabetic mice.

The PIV mechanism allows skin blood flow to increase in response to a nonpainful, locally applied pressure stimulation in humans (1) and rats (2). It has been reported that this cutaneous vasodilation involved C fibers, because it disappeared after chronic treatment with capsaicin in animals and humans. In addition, vasodilators such as the calcitonin gene-related peptide as well as endothelium factors prostaglandins and endothelial NO were involved in the PIV mechanism (2). In a previous study, we reported an early decrease of skin blood flow at low applied pressures in diabetic patients both with and without clinical neuropathy, compared with control subjects (3). In the present study, we observed an early decrease in LDF in response to external pressure application in the diabetic group that is similar to the one observed by Fromy et al. (3) in diabetic patients compared with control subjects. Indeed, LDF in 1-week diabetic mice was reduced beginning at a pressure as mild as 0.46 kPa, whereas LDF in control mice was not reduced until higher pressures (1.16 kPa). This clearly demonstrates the inability of the diabetic skin microcirculation to adapt to the applied pressure; as a result, 1-week diabetic mice exhibited no PIV responses compared with control mice. Under conditions of diabetic hyperglycemia, it has been suggested that three main biochemical pathways—nonenzymatic glycation, redox potential alterations, and the polyol pathway—decrease NO bioavailability (4,15). In our present study, advanced glycation end product accumulation was confirmed via fructosamine increase as early as 1 week of diabetes induction, suggesting a reduced NO activity (24,25). Increased flux of the polyol pathway is thought to competitively decrease NADPH, leading to impaired production of NO (26–30). Consumption of NADPH may also contribute to decrease the production of reduced glutathione and so decrease the antioxidant capacity leading to excessive oxidative stress and production of peroxinitrite (4,31). Therefore, the reduction in NO bioavailability that could occur as early as 1 week of diabetes could explain the absence of PIV, because NO was shown to be essential for its development (2).

To go further in the assessment of the cutaneous microvascular function in 1-week diabetic mice, we used the iontophoresis method, which gives the opportunity to study in vivo endothelium-dependent and -independent responses. Although this method is commonly used in diabetic patients (32–35), only a few diabetic animal studies have examined in vivo endothelium-dependent and -independent responses (36). We observed a reduced ACh-dependent relaxation in diabetic compared with control mice. ACh interacts with the endothelium and mediates its endothelium-dependent vasodilatory effect through a muscarinic receptor on the endothelial surface. This leads to a rise in intracellular calcium concentration, activation of NO synthase, and an increase in NO production. Therefore, it is recognized that an impairment of ACh-dependent relaxation mainly reflects a decrease in NO bioactivity or NO production. Thus, NO-dependent response reduction to ACh delivery in diabetic mice suggests that at 1 week of diabetes, there is already a reduction in endothelial NO levels, significant enough to impair PIV response, meaning that the process of diabetic endothelial alteration has already started. However, we cannot rule out possible alterations in other endothelial vasodilatory pathways contributing to the ACh response impairment such as endothelium-derived hyperpolarizing factor and prostanoids, which have also been proposed to mediate the effects of ACh in addition to NO (17).

In human studies, the iontophoresis technique has enabled clinical measurement of the effects of diabetes on vascular dysfunction. Several studies have demonstrated reduced endothelium-dependent vasodilation in patients with either type 1 or type 2 diabetes (32–35) that was concordant with our study. Vascular studies in animals using in vitro–isolated perfused arteries have provided inconsistent results with enhanced, unaltered, or impaired endothelium-dependent relaxation during diabetes (13–15) that might be explained by experimental conditions, including the type of artery and the type of vasodilator examined in a given artery preparation. However, our result is concordant with Terata et al. (37), who showed that ACh-induced vasodilation by arterioles, providing circulation to the region of the sciatic nerve, was impaired early in diabetes.

In contrast to ACh, SNP causes vasodilation by directly stimulating the vascular smooth muscle cells. The present data show that vasodilation resulting from iontophoretic application of SNP was not different between diabetic and control mice, thus demonstrating intact smooth muscle cell vasodilation capacity.

As expected, 1-week diabetic mice had no nerve alterations as shown by the similar MNCV, total fiber number, and myelinated fiber size between diabetic and control mice. These results are in agreement with several studies performed in rats (18,37,38) and mice (5). As nerve function was intact, it could not explain PIV absence in our mouse model.

Some authors (39) have observed a transient MNCV decrease in 1-week STZ-induced diabetic rats that came back to the onset level at 2 weeks. They suggested that the transient MNCV decrease could result from reduced ATP supply or related to changes in myo-inositol and phosphoinositide metabolism. We did not observe this change, which could be due to the animal model used. Our study used a diabetic mouse model, and the induction of diabetes and complications may be different from diabetic rat models. It is recognized that slowing of nerve conduction is a hallmark of both experimental and human diabetic neuropathy, and Coppey et al. (18) suggested that the vascular dysfunction observed in their study was responsible for the nerve disorders associated with the early onset of diabetes.

In conclusion, endothelium-dependent relaxation was reduced but still present, whereas PIV was totally abolished in 1-week experimental diabetic mice. These results are in accordance with those obtained in diabetic patients (40). These findings point out that PIV response seems to
be highly sensitive to endothelial NO levels, and therefore endothelial impairment by itself is sufficient to alter severely the PIV response. We suggest that PIV could be a sensitive indicator of endothelial alteration occurring during diabetes. PIV suppression could favor diabetes complications such as diabetic foot ulcers. Detecting and preventing early vascular dysfunction could delay the appara-
tion of diabetic neuropathy and limit diabetic complications. Further experiments are needed to explore the individual involvement of each biochemical pathway in PIV disappearance.

ACKNOWLEDGMENTS

D.S.-R. was supported by a grant from Conseil Régional des Pays de la Loire. The present study was supported by the French National Scientific Institute INSERM and the Region des Pays de la Loire (ESPRI 2002).

We thank the local Animal Care Unit of the University of Angers for facilities. We also thank Robert Filmon and Christine Audrin of the Microscopy-Histology free access platform for valuable advice and technical assistance. We are grateful to Dr. Gilles Simard for fructoseassay assays (Molecular Biology and Biochemistry department). We thank the laboratory of Neurobiology and transgenesis UPRES EA 3143.

REFERENCES


2. Fromy B, Merzeau S, Abraham P, Saumet JL: Mechanisms of the cutaneous vasodilator response to local external pressure application in rats: involve-
ment of CGRP, neurokinins, prostaglandins and NO. Br J Pharmacol 131:1161–1171, 2000


4. Brownlee M: Biochemistry and molecular cell biology of diabetic compli-


17. Peper GM: Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: import-


H1810, 1992


27. Soriano FG, Virig L, Jactap P, Szabo E, Mabley JA, Laret D, Marton A, Hoyt DG, Murthy KGK, Salzman AL, Southam GJ, Szabo C: Diabetic endothelial dysfunction: the role of poly (ADP-ribose) polymerase activa-


30. Cameron NE, Cotter MA, Basso M, Hohman TC: Comparison of the effects of inhibitors of aldose reductase and sorbitol dehydrogenase on neu-


