Comparison of skin microvascular reactivity with hemostatic markers of endothelial dysfunction and damage in type 2 diabetes

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Aim: Patients with non-insulin-dependent diabetes mellitus (NIDDM) are at increased cardiovascular risk due to an accelerated atherosclerotic process. The present study aimed to compare skin microvascular function, pulse wave velocity (PWV), and a variety of hemostatic markers of endothelium injury [von Willebrand factor (vWF), plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator (t-PA), tissue factor pathway inhibitor (TFPI), and the soluble form of thrombomodulin (s-TM)] in patients with NIDDM.

Methods: 54 patients with NIDDM and 38 sex- and age-matched controls were studied. 27 diabetics had no overt micro- and/or macrovascular complications, while the remainder had either or both. The forearm skin blood flow was assessed by laser-Doppler imaging, which allowed the measurement of the response to iontophoretically applied acetylcholine (endothelium-dependent vasodilation) and sodium nitroprusside (endothelium-independent vasodilation), as well as the reactive hyperemia triggered by the transient occlusion of the circulation.

Results: Both endothelial and non-endothelial reactivity were significantly blunted in diabetics, regardless of the presence or the absence of vascular complications. Plasma vWF, TFPI and s-TM levels were significantly increased compared with controls only in patients exhibiting vascular complications. Concentrations of t-PA and PAI-1 were significantly increased in the two groups of diabetics versus controls.

Conclusion: In NIDDM, both endothelium-dependent and -independent microvascular skin reactivity are impaired, whether or not underlying vascular complications exist. It also appears that microvascular endothelial dysfunction is not necessarily associated in NIDDM with increased circulating levels of hemostatic markers of endothelial damage known to reflect a hypercoagulable state.

Keywords: skin microcirculation, iontophoresis, pulse wave velocity, type 2 diabetes, hemostatic markers

Introduction

Endothelial cells have a strategic location at the interface between the blood and tissues and release a number of autocrine and paracrine substances critically involved in the regulation of vascular tone and the local control of flow and hemostasis (Verma and Anderson 2002). Insulin resistance and endothelial dysfunction are closely related during the development of type 2 diabetes (Caballero 2003; Kim et al 2006). In fact, endothelial dysfunction may be at the same time a cause and a consequence of the insulin resistance syndrome (Pinkney et al 1997; Wheatcroft et al 2003). Endothelial dysfunction and/or damage is associated with an increased cardiovascular risk and represents an early feature in the atherosclerotic process (Verma et al 2003; Brunner et al 2005).

The incidence of type 2 diabetes (non-insulin-dependent diabetes mellitus, NIDDM) is exploding worldwide and it is becoming a prominent burden on health

care (Murray and Lopez 1996; Amos et al 1997). Patients with NIDDM incur a high risk of cardiovascular and renal complications (Nathan 1993). The present investigation was performed to assess whether there is a graded worsening in endothelial function and/or damage from non-diabetic subjects to patients with NIDDM, regardless of whether micro- and/or macrovascular complications are exhibited.

Endothelial function was evaluated using a laser-Doppler flowmeter allowing the measurement of skin blood responses to iontophorized acetylcholine (Ach), which dilates by releasing nitric oxide (NO) from the endothelium, to sodium nitroprusside (SNP), which acts as a NO donor directly in vascular smooth muscle cells, and to local heating (Anderson 1999; Kubli et al 2000; Golay et al 2004). We also measured circulating levels of hemostatic regulatory molecules physiologically produced by endothelial cells, such as plasminogen activator-1 (PAI-1), von Willebrand factor (vWF), tissue plasminogen activator (tPA), free tissue factor pathway inhibitor (f-TFPI), and the soluble form of thrombomodulin (s-TM) (Cines et al 1998; Morange et al 2001, 2004). Increased plasma levels of these molecules may serve as markers of endothelial dysfunction and/or damage (Morange et al 2001; Blann et al 2002; Kato 2002; Kathiresan et al 2006). Finally, we determined pulse wave velocity as an index of arterial stiffness (Asmar et al 1995).

Methods

Subjects

We recruited 54 consecutive patients with type 2 diabetes referred to our outpatient clinic. The diagnosis of type 2 diabetes was based on the 1998 WHO criteria (Alberti and Zimmet 1998). Thirty-eight healthy subjects matched for gender and age served as controls. The latter had all a fasting glycemia <6.1 mmol/L, a body mass index (BMI) <30 kg/m^2 and a blood pressure $<140/90$ mmHg. They had no past history of heart, lung, kidney, endocrine, or liver disease and were not taking any medication. The protocol was approved by the Ethics Committee at our institution, and carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. Informed written consent was obtained from all subjects.

Our diabetic patients were routinely screened for macro- and microangiopathy (history of coronary heart disease, stroke or claudication, examination of carotid and peripheral arteries, and eye funduscopy by an ophthalmologist). In presence of 2 or more risk factors in addition to diabetes, a myocardial radionuclide scintigraphy or a stress echocardiography was performed (Consensus Development Conference on Insulin Resistance 1998). This was followed by a coronarography if required. Coronary heart disease was considered present if any one of the following criteria was met: history of transmural myocardial infarction, history of coronary angioplasty, history of coronary bypass surgery, positive evidence for coronary artery disease on an exercise tolerance test, a stress echocardiogram, a myocardial scintigraphy, or a coronary angiogram. Ultrasonic examinations of carotid and peripheral arteries were obtained where appropriate. Diabetic nephropathy was defined as an albumin/creatinine ratio >2 mg/mmol in a morning urine spot (Gerstein et al 2001; Jermendy et al 2001). Plasma levels of brain natriuretic peptide have been measured in the same population of diabetics and controls, and the results previously published (Beer et al 2005).

Protocol

All participants were studied on 2 mornings, after an overnight fast. The interval between the two sessions ranged from 1 to 14 days. On each study day the participants were asked to report at our research facility at 07.30, and were left to rest on a bed. They were not allowed to drink beverages containing caffeine, xanthine, or alcohol and were asked to abstain from smoking. The investigations were carried out in a quiet room with air conditioning. Ambient temperature was systematically measured.

Session 1: After a 30-minute resting period a venous cannula was inserted into an antecubital vein for blood sampling. This was done without the use of a tourniquet to avoid the activation of the coagulation cascade. A 10-mL blood sample was first collected in a prechilled sodium citrate-coated tube for determining circulating levels of PAI-1, vWF, tPA, f-TFPI, and s-TM. After centrifugation at 4°C, the plasma was stored at −80°C. An additional 10-mL blood sample was drawn in an heparinized tube for measurement of plasma, creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride concentrations.

Session 2: The outline of the study protocol is illustrated in Figure 1. On arrival a venous cannula was inserted in an antecubital vein and a 10-mL blood sample collected in a heparinized tube for measurement of plasma glucose, and insulin concentrations. Forearm skin temperature was assessed using a cutaneous probe (G. Mettraux, Crissier, Switzerland). Blood pressure and heart rate were measured in supine position using an automated oscillometric device (Datascope Accutor 1A, MS Cardio-Medical, Brunnen, Switzerland). At 08:00 the participants received a standard breakfast containing 75 g carbohydrates and 40 g

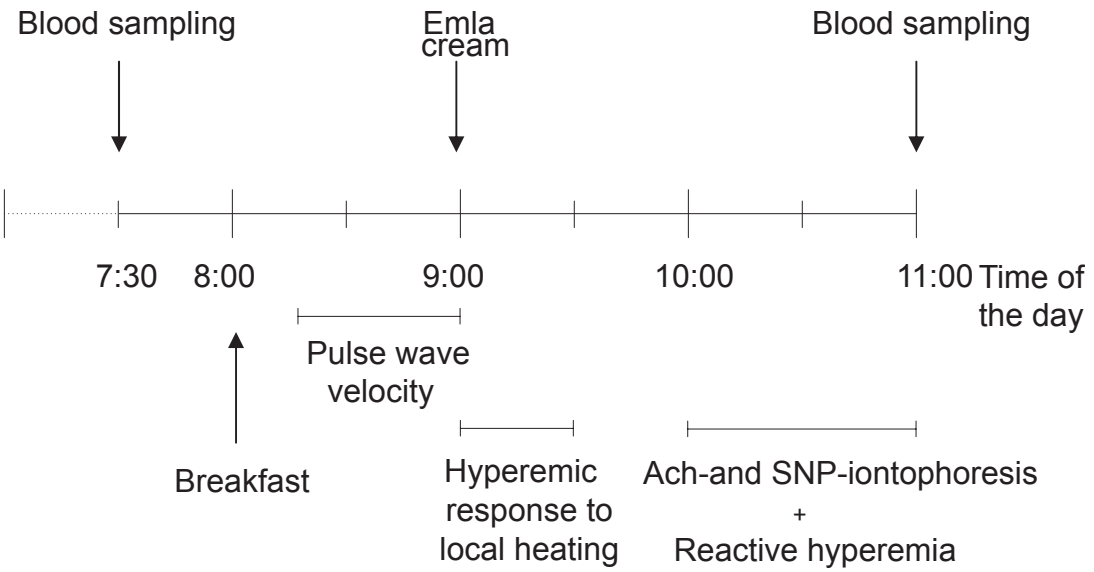

Figure 1 Outline of the study protocol.
Abbreviations: Ach, acetylcholine; SNP, sodium nitroprusside.

fat. Pulse wave velocity was determined between 08:15 and 09:00. The hyperemic responses to local heating of the forearm skin were then assessed, between 09:00 and 09:30. This was followed, between 10:00 to 11:00, by the measurement of the cutaneous microvascular responses to iontophorized (Ach and SNP), as well as to the transient interruption of forearm circulation (reactive hyperemia, RH). Blood sampling (10 mL) was repeated at the end of the session for determination of plasma glucose and insulin concentrations.

Measurement of pulse wave velocity (PWV)

The transmission velocity of the pulse pressure wave between the right carotid on one hand and the right femoral and radial arteries on the other hand was assessed using a validated, automatic device (Complior Artech Medicla) (Asmar et al 1995). The distance between the recording sites was determined with a tape-measure over the surface of the body. PWV was expressed in meters/sec.

Measurement of skin blood flow

A laser-Doppler imager (Moor Instruments, Axminister, UK) was used to measure skin blood flow, as described previously (Kubli et al 2000). This device allows the scanning of a region of interest, with no skin contact. The distance traveled by the incident laser beam from the laser aperture to the skin was set at 41 cm. The total flow is expressed in perfusion units (PU) according to the principle of laser-Doppler flowmetry. The sites chosen for the measurements were located on the proximal anterior face of the right forearm and were selected so as to exclude visible veins.

Response of skin blood flow to local heating

Two stainless steel, temperature-controlled, ring-shaped chambers with inner diameter, outer diameter, and thickness of 8, 25, and 8 mm, respectively, were affixed to the forearm skin with double-sided tape, filled to the rim with deonized water and overlaid with a transparent glass coverslip (Golay et al 2004). The skin underneath the coverslip and water was thus accessible to laser Doppler imaging, was programmed to repetitively scan the area comprising both chambers every 30 seconds.

The chambers were connected to analog temperature controllers with adjustable set-point. Their temperature was set at 34°C until a stable blood flow reading was obtained, then raised in 60 seconds to 37°C in one chamber and 41°C in the other, then maintained at this level for the next 11 minutes.

Response to iontophoretically applied Ach and SNP

Iontophoresis is a method of noninvasive transfer of charged molecules through the skin by means of an externally applied electrical current. To follow with laser Doppler flowmetry, the responses of skin blood flow to iontophoretically applied Ach and SNP, we constructed a ring-shaped chamber (10 mm internal diameter) in black neoprene fitted on the inside with an annular copper electrode connected to an iontophoresis controller (MIC1-e; Moor Instruments, Axminster, United Kingdom). The chamber was affixed to the skin with double-sided tape and filled to

the rim with a solution of either 1% Ach or 0.1% SNP in deionized water. The drug solutions were prepared fresh on each day (Ach, Sigma Chemie, Buchs, Switzerland; SNP, Schwarz Pharma, Aubonne, Switzerland). To avoid artifacts generated at the air – liquid interface, the chamber was covered with a thin, clear glass lid, with care taken to avoid trapping any bubbles underneath. The iontophoresis controller was also connected to an indifferent electrocardiograph electrode placed on the wrist. Polarity was adapted to the electric charge of the vasoactive molecule (chamber positive for Ach and negative for SNP). For testing Ach, 3 different doses of current charge density (1.4, 7, and 28 mC/cm^2) were administered, each in a pulsed fashion over 7 minutes, with 2 minutes interposed between each dose. For SNP, the current charge density was 38 mC (Kubli et al 2000). The skin was pretreated with an anesthetic cream (Emla cream 5%, Astra Pharmaceutica AG, Dietikon, Switzerland) applied for 1 hour under an occlusive dressing (Tegaderm, 3M Health Care Ltd, UK), in order to prevent current-induced, axon reflex-mediated vasodilation (Wardell et al 1993; Berghoff et al 2002).

Reactive hyperemia

Reactive hyperemia (RH) was assessed with the laser-Doppler imager in the forearm skin, at a site not exposed to local anesthesia. The arterial occlusion was achieved by a pressure cuff placed on the arm and inflated at a suprasystolic pressure (220 mmHg) for 3 minutes.

Laboratory tests

The plasma levels of vWF, PAI-1, tPA, f-TFPI, and s-TM were measured using commercially available enzyme-linked immunosorbent assays (Diagnostica Stago, Asnières, France).

Statistical analysis

Results are presented as means ± SD. Three groups of subjects (controls, diabetics without any vascular complication, and diabetics with microvascular and/or macrovascular complications) were compared with simple parametric analysis of variance. Where justified by very asymmetric distribution not amenable to logarithmic transform, a Kruskall-Wallis test was used instead. When the F (or chi-square) value was significant, pairwise comparisons were carried out with Dunn's test or its nonparametric adaptation (Glanz 1992). The alpha level of all tests was set at 0.05. All computations were performed with the JMP software, version 3.2.2 (SAS Institute, Cary, NC, USA).

Results

Characteristics of the study population

The baseline characteristics of the study subjects are summarized in Table 1. Diabetic patients were divided into 2 subgroups according to the presence (n = 27) or the absence of micro- and/or macrovascular complications (n = 27). Among the former patients, 9 exhibited microvascular complications and 5 macrovascular complications only, whereas 13 patients had both types of complications. Body mass index (BMI) as well as systolic and diastolic blood pressures were significantly higher in the two groups of diabetics than in the controls. Significant differences in mean levels of Hb1Ac, fasting and post-prandial plasma glucose, fasting plasma insulin, plasma creatinine, and urinary albumin/creatinine ratio were observed between the two groups of diabetics. An exercise tolerance test, a coronary angiogram, a stress echocardiogram, and a myocardial radionuclide scintigraphy were performed in 6, 5, 24, and 11 patients, respectively. All patients who underwent the echocardiographical examination showed an ejection fraction above 50%.

Table 2 shows the various treatments received at the time of examination. Blockers of the renin-angiotensin system, β-blockers, and anti-aggregatory agents were taken significantly more often in patients with than in those without vascular complications.

There was no significant difference in room and skin temperature between the 3 study groups (Table 3).

Measurement of pulse wave velocity (Table 3)

Carotid to radial PWV was comparable in the control subjects and in the two groups of diabetics. Diabetic patients with vascular complications showed significantly increased values for carotid to femoral PWV compared with both control subjects and diabetic patients without vascular complications.

Measurement of skin blood flow (Table 3)

No significant difference in baseline skin blood flow was observed between the control subjects and the two groups of diabetics regardless of whether the skin had been exposed to the anesthetic cream.

Response of skin blood flow to local heating (Table 3)

The maximal skin microvascular dilation induced by increasing the local temperature to 37°C was of similar

Table 1 Characteristics of study participants

	Controls	Diabetics	
		without vascular complications	with vascular complications
Number of subjects	38	27	27
Duration of diabetes (years)	–	6.4 ± 6.2	10.8 ± 7.8
Age (years)	56.0 ± 11.1	54.4 ± 7.7	57.9 ± 7.0
Sex (M/F)	25/13	16/11	22/5
Body mass index (kg/m^2)	23.0 ± 2.5	30.4 ± 4.8[b]	31.4 ± 5.4[b]
Waist to hip ratio	0.92 ± 0.07	1.01 ± 0.05[b]	1.04 ± 0.08[b]
Blood pressure, systolic (mmHg)	117 ± 10	133 ± 17[b]	142 ± 16[b]
Blood pressure, diastolic (mmHg)	68 ± 8	78 ± 11[b]	82 ± 9[b]
Smoking (pack-years)	1.7 ± 7.2	9.7 ± 23.0[a]	8.5 ± 13.5[a]
Plasma glucose, fasting (mM)	4.8 ± 0.5	7.0 ± 1.6[b]	8.4 ± 2.2[bc]
Plasma glucose, post-prandial (mM)	5.1 ± 0.9	12.4 ± 2.4[b]	14.2 ± 2.6[bc]
Plasma insulin (mU/L)			
fasting	10.9 ± 5.3	23.5 ± 13.2[b]	35.4 ± 21.7[bc]
post-prandial	40.5 ± 23.6	79.4 ± 50.7[b]	82.2 ± 48.8[b]
HOMA-IR index	2.3 ± 1.3	7.5 ± 5.2[b]	13.5 ± 9.7[bc]
HbA1c (%)	NA	7.3 ± 1.1	8.2 ± 1.3[c]
HDL-cholesterol (mM)	1.62 ± 0.34	1.29 ± 0.36[b]	1.30 ± 0.30[b]
LDL-cholesterol (mM)	2.96 ± 0.89	2.85 ± 0.71	2.75 ± 0.87
Triglycerides (mM)	0.94 ± 0.47	1.87 ± 0.80[b]	2.09 ± 1.92[b]
Plasma creatinine (μM)	89 ± 10	87 ± 15	104 ± 26[bc]
Urine albumin/creatinine ratio[a]	0.6 ± 0.5	0.8 ± 0.6	32.4 ± 83.9[bc]

Notes: Means ± SD. NA, not available.
Statistical analysis: one way parametric ANOVA except for smoking (Kruskall-Wallis non parametric ANOVA).
[a]$p < 0.05$ vs controls; [b]$p < 0.01$ vs controls; [c]$p < 0.01$ diabetics with vs diabetics without vascular complications.

magnitude in the three study groups. However, when the skin temperature was increased to 41°C, maximal vasodilation was significantly lower in diabetics than in controls. The two diabetic groups did not differ in this respect.

Response to iontophoretically applied Ach and SNP (Table 3)

Three different doses of current charge density were tested for the iontophoresis of Ach. The peak vasodilatory responses to the 1.4 mC/cm^2 and 7 mC/cm^2 charges were comparable in the thee groups. With the highest 28 mC/cm^2 charge, however, a significant attenuation of the responses was observed in the two groups of diabetics compared with controls. The responses were equally blunted in the diabetics with and without vascular complications. The peak responses to iontophorized SNP were significantly attenuated in the two groups of diabetes, with no difference, however in the presence and the absence of vascular complications.

Reactive hyperemia (Table 3)

The peak hyperemic response was significantly lower in diabetics than in controls, to a similar extent in patients with and without vascular complications.

Laboratory tests (Table 4)

In diabetics without complications, the plasma levels of PAI-1 and t-PA were elevated to the same extent as in those with complications, whereas the levels of the three other markers were normal. In diabetic patients with vascular complications, the plasma concentrations of all hemostatic endothelial cell markers were significantly increased compared with control subjects.

Discussion

Type 2 diabetes mellitus is becoming increasingly prevalent in both developing and industrialized countries, representing therefore a leading cause of health disability and health care cost (Murray and Lopez 1996; Amos et al 1997). NIDDM is

Table 2 Number of patients (%) on various therapies

	Diabetics without vascular complications	Diabetics with vascular complications	
Insulin	13 (48%)	19 (70%)	NS[a]
Oral antidiabetics	19 (70%)	15 (56%)	NS
ACE-I or AT_1-receptor blocker	14 (52%)	21 (78%)	<0.05
β-blocker	0	12 (44%)	<0.001
Diuretic	8 (30%)	13 (48%)	NS
Calcium antagonist	7 (26%)	9 (33%)	NS
Statin	10 (37%)	16 (59%)	NS
Anti-aggregatory agents	9 (33%)	24 (89%)	<0.001

[a]not significant
Abbreviation: ACE-I, angiotensin converting enzyme inhibitor.

a strong risk factor not only for nephropathy and end stage renal failure, but also for all manifestations of atherosclerotic disease (Nathan 1993). Most patients with NIDDM die from cardiovascular complications (Laasko and Lehto 1997), which can be explained by the fact that NIDDM is frequently associated with other risk factors such as hypertension, central obesity, hyperinsulinemia, and dyslipidemia, the last named being characterized mainly by elevated serum total triglycerides and low HDL cholesterol, ie, components of the metabolic syndrome (Reaven et al 1996; Pyorala et al 2000; Waeber et al 2001). Not surprisingly most of our patients treated for NIDDM received also medications for high blood pressure and dyslipidemia (Table 2). Of note also is the fact that our patients, whether or not they had detectable vascular complications, had increased waist to hip ratio, plasma insulin, glucose and triglyceride levels, and decreased plasma HDL cholesterol levels compared with age- and sex-matched healthy controls (Table 1). The patients with

Table 3 Pulse wave velocity and microvascular skin blood flow responses in diabetic patients with and without vascular complications compared with control subjects (means ± SD)

	Controls	Diabetics	
	(n = 38)	without complications (n = 27)	with complications (n = 27)
Room temperature (°C)	24.0 ± 0.8	24.3 ± 1.1	24.1 ± 0.7
Skin temperature (°C)	32.7 ± 1.1	32.4 ± 1.2	32.8 ± 1.0
Baseline skin blood flow (PU)			
anesthetized skin	60 ± 13	60 ± 10	63 ± 13
unanesthetized skin	89 ± 28	74 ± 20	89 ± 30
Pulse wave velocity (m/sec)			
carotid-femoral artery	9.4 ± 2.2	9.9 ± 1.8	11.8 ± 2.2[b,c]
carotid-radial artery	10.2 ± 1.9	9.8 ± 1.7	10.0 ± 0.9
Maximal response to local heating (PU)			
37°C	52 ± 45	54 ± 41	56 ± 40
41°C	322 ± 130	257 ± 111[a]	242 ± 112[a]
Maximal response to Ach (PU)			
1.4 mC/cm^2	229 ± 119	211 ± 139	163 ± 94
7.0 mC/cm^2	362 ± 89	317 ± 115	301 ± 115
28.0 mC/cm^2	395 ± 94	345 ± 99[a]	337 ± 102[a]
Maximal response to SNP (PU)	326 ± 80	274 ± 86[b]	255 ± 65[b]
Maximal RH (PU)	183 ± 67	136 ± 55[a]	134 ± 69[a]

Analysis with one way parametric ANOVA
[a]$p < 0.05$ vs controls; [b]$p < 0.01$ vs controls; [c]$p < 0.01$ vs diabetics without complications.
Abbreviations: Ach, acetylcholine; PU, perfusion units; RH, reactive hyperemia; SNP, sodium nitroprusside.

Table 4 Plasma concentrations of hemostatic endothelial cell markers in diabetic patients with and without vascular complications compared with control subjects (means ± SD)

	Controls	Diabetics	
	(n = 38)	without complications n = 27)	with complications n = 27)
PAI-1 (ng/mL)	13.7 ± 7.5	51.1 ± 37[b]	50.0 ± 40.4[b]
tPA (ng/mL)	6.2 ± 4.1	10.9 ± 4.4[b]	10.5 ± 4.4[b]
vWF (IU/dL)	136 ± 48	145 ± 50	180 ± 65[bc]
f-TFPI (ng/mL)	7.6 ± 3.9	7.8 ± 2.5	9.6 ± 2.5[ac]
s-TM (ng/mL)	56.8 ± 30.5	53.4 ± 16	77.1 ± 46.7[ac]

Statistical analysis: one way parametric ANOVA except for PAI-1 (Kruskall Wallis non parametric ANOVA)
[a]$p < 0.05$ vs controls; [b]$p < 0.01$ vs controls; [c]$p < 0.05$ vs diabetics without complications.
Abbreviations: PAI-1, plasminogen activator inhibitor-1; vWF, von Willebrand factor; tPA, tissue plasminogen activator; f-TFPI, free tissue pathway factor inhibitor; s-TM, soluble form of thrombomodulin.

vascular complications showed significantly higher plasma glucose, insulin, and HbA1c levels, as well as significantly higher systolic blood pressure than patients without vascular complications. These differences were seen despite a very similar pattern of drug prescriptions in the two groups, except a substantially greater proportion of patients on antiplatelet therapy among individuals with vascular complications (Table 2). The poorer glucose and blood pressure control achieved might have accelerated the atherosclerotic vascular disease observed in the latter patients. Whether the presence of vascular complications made our patients with NIDDM less responsive to antihypertensive therapy is another possibility to be considered.

Impaired endothelial function, as reflected by a decreased endothelium-dependent vasodilation, is a hallmark of vascular disease states (Brunner et al 2005). A common denominator to all cardiovascular risk factors is an increased oxidative stress, resulting in the scavenging of NO, the potent relaxing factor released by the endothelium (Waeber and Feihl 2005; Feletou and Vanhoutte 2006. This has been repeatedly documented in patients with NIDDM (Calles-Escandon and Cipolla 2001; Ding and Triggle 2005). Endothelial dysfunction refers usually to a blunted vasodilatory response to Ach, which releases NO from the endothelium, while the vasorelaxant activity of SNP or nitroglycerin, which act as NO donors in vascular muscle cells, is maintained (Deanfield et al 2005). Another way of testing endothelial function is to assess the flow-mediated vasodilation, ie, the vasodilation occurring after transient occlusion of the circulation (Anderson 1999; Deanfield et al 2005). Still another means to evaluate endothelium function in humans is to study the vasodilation induced by local skin heating (Kellogg et al 1999).

Both the endothelium-dependent and -independent blood flow responses (measured using venous occlusion plethysmography or ultrasonography) have been reported to be attenuated in the forearm circulation of patients with NIDDM (Zenere et al 1995; Williams et al 1996; Hogikyan et al 1998; Natali et al 2006). In three of these studies, hypertensive and dyslipidemic patients were excluded (Zenere et al 1995; Williams et al 1996; Hogikyan et al 1998), whereas in the fourth, 40% and 10% of patients had hypertension (either treated or untreated) or dyslipidemia (treated with a fibrate), respectively (Natali et al 2006). Notably, in Williams et al (1996) study, measurements were performed after administration of aspirin to inhibit the endogenous production of vasoactive prostanoids from the endothelium. Impaired vasodilatory responses to Ach and SNP have also been reported in the skin microvasculature of type 2 diabetics (Morris et al 1995; Lim et al 1999; Caballero et al 1999. Ach and SNP were applied transcutaneously by iontophoresis and skin blood flow changes measured by laser Doppler imaging. Patients receiving blood pressure- or lipid-lowering drugs were excluded. We made similar observations in the present investigation, as both the Ach and SNP responses were blunted in the presence of NIDDM. It has to be stressed that most of our patients were taking not only antidiabetic medications, but also blood pressure- and lipid-lowering agents, as well as anti-aggregatory drugs. The need for this polypharmacy in high risk patients with NIDDM has been well documented recently (American Diabetes Association 2001; Waeber et al 2001). An original aspect of our investigation deals therefore with the finding of a persistently impaired microvascular reactivity in type 2 diabetes despite efforts directed to control all traditional cardiovascular risk factors. Also, blockers of the renin-angiotensin system and statins were the treatments of choice in our patients, which corresponds to the current recommendations for the pharmacological management of hypertension and dyslipidemia in

patients with NIDDM (American Diabetes Association 2001; Waeber et al 2001, 2003; European Society of Hypertension 2003). The lack of normalization of skin microvascular reactivity in our patients might have been expected since impaired responses to iontophoretically applied Ach and SNP have been reported in subjects with impaired glucose tolerance or increased fasting glucose, already before the development of established type 2 diabetes (Caballero et al 1999; Vehkavaara et al 1999).

In addition to the reduced vasodilatory response to iontophorized Ach and SNP, we observed a significant attenuation of the hyperemia caused by local heating of the skin. This is in agreement with the previous experience of other investigators (Vinik et al 2001). Of note is that the abnormal skin response to local heating may reflect not only endothelial dysfunction, but also the impaired reactivity to nociceptive stimulation and the alterations in neuronal release of vasoactive peptides encountered in type 2 diabetes (Stansberry et al 1999; Vinik et al 2001). Interestingly, the limited microvascular vasodilation triggered by raising the skin temperature had already been observed in patients at risk of developing NIDDM, as manifested by elevated plasma glucose concentrations still below the diabetic range (Jaap et al 1994, 1997).

The last method we used to explore the endothelial function of the skin forearm microcirculation was the measurement of the flow-mediated vasodilatory capacity, done by assessing the reactive hyperemia that occurs after the release of a transient interruption of the circulation in the brachial artery. A blunted response was evidenced in the diabetics, as expected from previous observations of brachial artery diameter changes during the post-ischemic period (Caballero et al 1999).

All methods we used for endothelial function testing showed an impaired reactivity of skin microcirculation. Our diabetic patients were subdivided in 2 groups according to the presence or absence of overt micro- and/or macrovascular complications. It was hypothesized that endothelial function would be altered to a greater degree in patients with manifest vascular damage. This was, however, not the case: there was no way to discriminate between patients with and without vascular complications based on any of the functional markers that were measured. By contrast, PWV, which is known to closely reflect arterial thickness (Laurent et al 2006), was on average higher in patients with than in those without vascular complications, while no difference was detected between the latter patients and control subjects. Increased arterial stiffness might occur before the onset of NIDDM (Henry et al 2003) and has an independent predictive value for mortality already at the stage of glucose intolerance (Cruickshank et al 2002). Among our diabetic patients, only those with clinical micro- and/or macrovascular disease exhibited higher PWV in comparison with control subjects. In theory, functional alterations of the endothelium should occur before structural changes of the arterial wall. Such a sequence in the atherosclerotic process could still be detected in our patients, even if they took a number of medications for controlling hyperglycemia, high blood pressure and/or dyslipidemia.

The endothelium is perturbed at all stages of atherogenesis: functional abnormalities are seen early during the course of the arterial wall disease, which is followed by a progressive injury of endothelial cells as a result of the underlying inflammatory process and the development of a prothrombotic state (Ross 1999; Libby and Simon 2001). The concept of "vulnerable blood" has been proposed to reflect the hypercoagulability associated with atherosclerosis and the predominant release of procoagulant factors occurring which characterizes this condition (Naghavi et al 2003). Increased circulating levels of vWF, PAI-1, t-PA, TFPI, and s-TM have been reported in patients with NIDDM (Collier et al 1992; McGill et al 1994; Bagg et al 2001; Aso et al 2002; Leurs et al 2002; Rigla et al 2006). In our study, plasma concentrations of all these markers of endothelial suffering were significantly higher in patients with vascular complications than in control subjects. In the absence of vascular complications, however, only plasma t-PA and PAI-1 levels were significantly increased compared with those in non-diabetic controls. Notably, the presence of vascular or renal complications has been found in relation to an increased propensity to develop coagulation and fibrinolysis abnormalities (Kario et al 1995; Yano et al 2003; Erem et al 2005). Our findings are relevant as they show that endothelial dysfunction in NIDDM is not necessarily accompanied by a clustering of hemostatic abnormalities, as would be expected if endothelial cells were damaged. The fact that most of our patients received medications known to have a protective action on the endothelium might have contributed to this situation.

As a final and practical note, the apparent progression seen in our data from normal plasma levels of vWF, f-TFPI and s-TM as well as a normal PWV in diabetics without vascular or renal complications, to generalized abnormalities of these parameters when such complications are detected, suggests that the serial measurement of some, or possibly all, of these parameters might be useful for the long term monitoring of these patients. Longitudinal studies would be required to verify this point.

In summary, our data show that patients with NIDDM, when treated as required for coexisting risk factors, exhibit endothelial dysfunction at the level of the skin microvasculature. This functional abnormality is associated with increased levels of some, but not all, hemostatic markers of endothelial damage as long as there is no concurrent micro- and/or macrovascular complication. Further studies are however needed to assess whether the control of all cardiovascular risk factors allows the prevention and/or the regression of endothelial damage in patients with type 2 diabetes.

Disclosures

None of the authors have conflicts of interest to declare.

Abbreviations

Ach, acetylcholine; NIDDM, non-insulin-dependent diabetes mellitus; PAI-1, plasminogen activator inhibitor-1; PU, perfusion units; RH, reactive hyperemia; SNP, sodium nitroprusside; tPA, tissue plasminogen activator; vWF, von Willebrand factor; f-TFPI, free tissue pathway factor inhibitor; s-TM, soluble form of thrombomodulin.

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