Endothelium-Dependent Vasodilatation, Plasma Markers of Endothelial Function, and Adrenergic Vasoconstrictor Responses in Type 1 Diabetes Under Near-Normoglycemic Conditions

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It is unknown whether and to what extent changes in various endothelial functions and adrenergic responsiveness are related to the development of microvascular complications in type 1 diabetes. Therefore, endothelium-dependent and endothelium-independent vasodilatation, endothelium-dependent hemostatic factors, and one and two adrenergic vasoconstrictor responses were determined in type 1 patients with and without microvascular complications. A total of 34 patients with type 1 diabetes were studied under euglycemic conditions on two occasions (11 without microangiopathy, 10 with proliferative and preproliferative retinopathy previously treated by laser coagulation, 13 with microalbuminuria, and 12 healthy volunteers also were studied). Forearm vascular responses to brachial artery infusions of N^G-monomethyl-L-arginine (L-NMMA), sodium nitroprusside, acetylcholine (ACh), clonidine, and phenylephrine were determined. The ACh infusions were repeated during coinfusion of L-arginine. Furthermore, plasminogen activator inhibitor type 1 (PAI-1) activity, tissue plasminogen activator antigen levels, von Willebrand factor antigen levels, tissue factor pathway inhibitor (TFPI) activity, and endothelin-1 levels were measured. No differences in endothelium-dependent or endothelium-independent vasodilatation or adrenergic constriction were observed between the diabetic patients and the healthy volunteers. In comparison to the first ACh infusion, the maximal response to repeated ACh during L-arginine administration was reduced in the diabetic patients, except in the patients with proliferative and preproliferative retinopathy previously treated by laser coagulation. In these patients, the combined infusion of L-arginine and ACh resulted in an enhanced response. TFPI activity was elevated, and PAI-1 activity was reduced in the type 1 diabetic patients. Furthermore, PAI-1 activity was positively correlated with urinary albumin excretion ($r = 0.48$, $P < 0.01$) and inversely correlated with the vasodilatory response to the highest ACh dose ($r = –0.37$, $P < 0.05$). The response to the highest ACh and L-NMMA dose were positively correlated with mean arterial blood pressure ($r = 0.32$, $P < 0.01$; $r = 0.41$, $P < 0.01$, respectively). Forearm endothelium-dependent and endothelium-independent vasodilatation and adrenergic responsiveness were unaltered in type 1 diabetic patients with and without microvascular complications. Relative to healthy control subjects, endothelium-dependent vasodilatation was depressed during a repeated ACh challenge (with L-arginine coinfusion) in the diabetic patients without complications or with microalbuminuria. In contrast, this vasodilatation was enhanced in the patients with retinopathy. Elevation of TFPI was the most consistent marker of endothelial damage of all the endothelial markers measured. *Diabetes* 47:1300–1307, 1999

Vascular complications are the main causes of morbidity and mortality in patients with diabetes. Several lines of evidence suggest that endothelial damage could play a key role in the development of both micro- and macroangiopathy. Animal studies suggest that diabetes or even hyperglycemia, per se, can result in impaired endothelium-dependent vasodilatation by reduced bioavailability of endothelium-derived NO (1). Loss of NO would result in enhanced contractility and proliferation of vascular smooth muscle cells, as well as increased platelet aggregation and adhesion (2). In type 1 diabetic patients, both normal and impaired endothelium-dependent vasodilatation determined by different techniques in different vascular beds have been reported (3–12). Also, the association between diabetic (incipient) nephropathy and endothelium-dependent vasodilatation is unclear. Elliot et al. (5) found impaired endothelium-dependent vasodilatation in patients with microalbuminuria. However, in patients with macroal-

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ACh, acetylcholine; ACh30, 30 g/min acetylcholine; C–, patients without complications; CV, coefficient of variation; EDHF, endothelium-derived hyperpolarizing factor; ELISA, enzyme-linked immunosorbent assay; ET-1, endothelin 1; FBF, forearm blood flow; L-NMMA, N^G-monomethyl-L-arginine; L-NMMA8, 8 mmol/min N^G-monomethyl-L-arginine; MA+, microalbuminuria with or without retinopathy; MAP, mean arterial pressure; PAI-1, plasminogen activator type 1; R+, proliferative and preproliferative retinopathy previously treated by laser coagulation; TFPI, tissue factor pathway inhibitor; t-PA, tissue plasminogen activator; UAE; urinary albumin excretion; vWF, von Willebrand factor.

buminuria, normal endothelium vasodilatation was reported in another recent study (8). These differences could be based on factors such as patient selection, duration of disease, ambient glucose levels, and concomitant abnormalities such as hypercholesterolemia or elevated blood pressure (13). In one study that included type 1 diabetic patients with elevated LDL cholesterol levels, vascular reactivity was impaired, which correlated with LDL cholesterol levels (10).

NO is formed from L-arginine by NO synthase, and the impaired endothelium-dependent vasodilatation can be restored by exogenous L-arginine in aortic rings of streptozotocin-induced diabetic rats (14,15); although in diabetic hamsters, no such improvement by L-arginine was observed (16). However, information on the effect of exogenous L-arginine on endothelium-dependent vasodilatation in patients with microvascular complications is scarce. Furthermore, in patients with diabetic retinopathy, endothelium-dependent vasodilatation has to our knowledge not been studied. Therefore, the first aim of the present study was to determine basal and stimulated endothelium-dependent vasodilatation in type 1 diabetic patients with microalbuminuria and/or retinopathy and to explore whether this vasodilatation can be enhanced by L-arginine administration.

Although several studies have reported on abnormalities in circulating endothelium-derived factors, which are involved in hemostasis and fibrinolysis and considered to be markers of generalized endothelial damage or endothelial dysfunction (1,17–19), it is not well known whether endothelial hemostatic and vasomotor disturbances occur in concert in diabetes. Therefore, the second aim was to study the interrelationship between these two aspects of endothelial function.

Finally, besides the vasoactive agents produced by the endothelium, sympathetic adrenergic vasoconstrictor nerves are an important control mechanism of local blood flow. Reduced endothelium-dependent vasodilatation can result in increased α_2 responsiveness (20). Indeed, in an earlier study in type 1 diabetic patients with microalbuminuria, we found enhanced venous constrictor responses to local infusion of clonidine (stimulates mainly α_2 adrenoceptors), while the venous sensitivity to phenylephrine (mainly α_1 adrenoceptor) appeared to be normal (21). Whether α_2 adrenoceptor sensitivity is also enhanced in arterial vessels is presently unknown. Thus, the third aim of our study was to assess the vasoconstrictor response of the forearm vasculature to α_1 and α_2 agonists in type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

Subjects. Experiments were performed in 34 type 1 diabetic patients and 12 age- and sex-matched healthy volunteers. Patients were recruited from the outpatient clinics of participating hospitals, and control subjects were recruited by advertisements in local papers. Three subgroups of diabetic patients could be formed: 11 patients without complications (C–), 10 with proliferative and preproliferative retinopathy previously treated by laser coagulation (R+), and 13 with microalbuminuria with or without retinopathy (MA+). Microalbuminuria was defined as urinary albumin excretion (UAE) of 30–300 mg/24 h (mean of two samples). Supine blood pressure did not exceed 160/90 mmHg in any of the individuals, and none of them had clinical signs or symptoms of atherosclerosis or neuropathy. Lipid levels were normal and serum creatinine levels were <100 µmol/l. The subjects did not use any medication (including non-steroid anti-inflammatory drugs or ACE inhibitors) for at least 4 weeks preceding the study, except for insulin in the diabetic patients. The study was approved by the Medical Ethical Committee of the University Hospital Maastricht, and all subjects gave written informed consent.

Protocol. All experiments were performed in a quiet, temperature-controlled room (24–25°C) after an overnight fast. Subjects were studied twice, on day A and day B, with an interval of at least 2 weeks in men or one complete menstrual cycle in women. All female subjects were studied in the follicular phase of the menstrual cycle. Subjects were not allowed to smoke, drink (except for water), or eat for at least 10 h before the experiment. They were studied in the supine position and remained in bed during the experiments. On the morning of the experiments, the diabetic patients omitted their insulin injection. After local anesthesia with lidocaine, a 20-gauge artery catheter was inserted retrogradely into the brachial artery of the nondominant arm for blood sampling and blood pressure recordings. The catheter was kept patent by 0.9% saline. In the diabetic patients, an additional catheter was inserted into an antecubital vein of the contralateral arm for infusion of insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) and/or 5% glucose to keep blood glucose levels between 4 and 8 mmol/l. The amount of insulin infused was based on the individual daily insulin dose. Experiments started when near-normoglycemia (4.0–8.0 mmol/l) had lasted for at least 30 min. Basal endothelial NO production was assessed by the vasoconstrictor response to local infusion of N^G-monomethyl-L-arginine (L-NMMA), a blocker of NO synthase. Stimulated endothelium-dependent vasodilatation was assessed by the response to acetylcholine (ACh).

On day A, the vasoconstrictor response of the forearm vasculature to clonidine, phenylephrine, and L-NMMA was studied. Clonidine (Boehringer, Ingelheim, Germany) in incremental doses of 0.05, 0.5, and 5.0 µg/min and phenylephrine (Centrafarm, Etten-Leur, the Netherlands) in incremental doses of 0.3, 1.0, and 3.0 µg/min were infused intra-arterially (each dose for 4 min). The sequence of the clonidine and phenylephrine infusions was randomized, each with a washout period of 75 and 60 min, respectively. After the second washout period, L-NMMA (Clinalfa, Läufelfingen, Switzerland) was infused at rates of 1, 2, and 8 µmol/min (each dose for 5 min).

On day B, the vasodilatory responses to ACh (Dispersa AG, Hettlingen, Switzerland) in doses of 3, 10, and 30 µg/min and sodium nitroprusside (Roche, Mijdrecht, the Netherlands) in doses of 1, 3, and 10 µg/min (each dose for 3 min) were measured. ACh and sodium nitroprusside were infused intra-arterially in randomized order, each with a washout period of 30 min. At 30 min after the second washout period, all hemodynamic measurements were repeated, and subsequently L-arginine (Fresenius, Bad Homburg, Germany) was infused intra-arterially at a dose of 10 mg/min for 20 min. Immediately thereafter, the three doses of ACh were infused again under continuation of the L-arginine infusion. Day A and day B were randomized.

All drugs were dissolved in saline, except for sodium nitroprusside and L-NMMA. Sodium nitroprusside was dissolved in 5% glucose, and L-NMMA was dissolved with mannitol (1.54%) in saline because L-NMMA was purchased in ready-to-use aliquots, which had been freeze-dried with mannitol. Fresh solutions were prepared just before each set of measurements. Dose, duration, and washout periods of the clonidine and phenylephrine infusions were based on pilot experiments in healthy control subjects. In these pilot studies, clonidine and phenylephrine consistently induced forearm vasoconstriction, which was maximal after 3 min, and which at the highest dose employed resulted in a response on the flat part of the dose-response curve in healthy subjects. All other infusions were based on data obtained in earlier studies (22,23). The duration of drug infusions was such as to reach a steady state in the vascular responses.

Hemodynamic measurements. Forearm blood flow (FBF) was determined in both arms simultaneously using electrocardiogram-triggered strain gauge venous occlusion plethysmography (Periflow, Janssen Scientific Instruments, Beerse, Belgium) (22). The hand circulation was excluded during FBF measurements by inflating a wrist cuff to suprasystolic pressure, starting 1 min before each FBF measurement. Hence, FBF measurements represent, predominantly, muscle blood flow. FBF was assessed during 3 min of baseline blood flow and again during local infusions of three cumulative doses of vasoactive drugs. The mean FBF value of the last minute of each drug step was used for calculations. Responses to the various drugs were calculated as percentage change in the FBF ratio (FBF infused arm divided by FBF in the contralateral arm). This calculated ratio ensures that only the direct effects of locally infused substances on FBF are taken into account. Blood pressure was measured through the intra-arterial catheter (Arrow, Reading, U.K.), using a Hewlett Packard 78205C monitor (Boeblingen, Germany). Heart rate was derived from the ECG. All signals were stored on the hard disk of a personal computer by means of a custom-built data acquisition system.

Blood sampling and assays. Venous blood samples for determination of serum total cholesterol, HDL cholesterol, triglycerides, plasminogen activator type 1 (PAI-1) activity, tissue plasminogen activator (t-PA) antigen, von Willebrand factor (vWF) antigen, tissue factor pathway inhibitor (TFPI) activity, and endothelin 1 (ET-1) were drawn just before the first hemodynamic measurements on day B (see PROTOCOL section). Blood glucose levels were determined every 5 min with adjustment of the intravenous insulin infusion, until steady-state glucose levels were between 4 and 8 mmol/l. At the steady state, arterial glucose samples were drawn every 30 min. Glucose was determined instantaneously by the glucose oxidase–based method (YSI, Yellow Springs, OH) in arterial whole blood. Blood samples for PAI-1, t-PA, vWF, and TFPI were collected in tubes containing 3.25% sodium citrate (dilution 1:10) and spun immediately at 5,000*g* for 10 min at 4°C. PAI-1 activity was measured photometrically (Kabi Diagnostica, Sweden; intra-assay coefficient of variation [CV] 6.0%; normal range 1–20 arbitrary units/ml). t-PA antigen was determined by an enzyme immunoassay (Innogenetics,

TABLE 1
Baseline characteristics

	Healthy volunteers	C–	R+	MA+
M/F	10/2	8/3	9/1	10/3
Smokers (*n*)	5	3	3	5
Age (years)	31 (33–47)	32 (25–45)	38 (32–49)	38 (28–40)
BMI (kg/m^2)	23.3 (22.8–24.1)	22.7 (20.6–23.7)	23.6 (22.9–25.1)	24.3 (23.7–25.5)
Diabetes duration (years)	—	13 (11–22)	19 (17–23)	20 (17–26)
HbA_{1c} (%)	—	8.4 (6.8–9.0)	8.8 (8.2–9.0)	9.6 (7.8–10.1)
Glucose (mmol/l)	4.3 (4.2–4.4)	7.1 (5.6–7.4)*	6.7 (6.4–7.5)*	7.5 (6.7–7.9)*
Total cholesterol (mmol/l)	4.6 (3.5–5.3)	4.3 (4.0–5.4)	4.7 (4.1–5.1)	4.1 (3.5–4.5)
HDL cholesterol (mmol/l)	1.2 (1.0–1.3)	1.4 (1.2–1.6)	1.1 (0.7–1.4)	1.0 (0.9–1.3)
Triglycerides (mmol/l)	1.45 (0.90–1.72)	0.82 (0.66–1.08)†	0.77 (0.57–1.07)†	0.78 (0.50–0.96)†
UAE (mg/24 h)	—	11 (5–16)	11 (5–16)	150 (70–431)‡
Noradrenaline (nmol/l)	0.61 (0.51–0.81)	0.63 (0.39–0.95)	0.71 (0.66–0.77)	0.72 (0.57–0.81)
Systolic blood pressure (mmHg)	107 (103–122)	106 (98–117)	108 (100–133)	123 (116–140)§
Diastolic blood pressure (mmHg)	70 (62–74)	67 (61–84)	75 (65–79)	80 (72–86)
MAP (mmHg)	81 (75–85)	80 (77–84)	87 (80–92)	94 (88–99)§
Heart rate (beats/min)	53 (51–59)	67 (58–70)	66 (63–69)*	74 (64–83)*
FBF (ml · 100 ml^{-1} · min^{-1})				
Infused arm	2.8 (2.1–3.2)	2.5 (2.2–4.1)	3.0 (1.9–3.9)	2.8 (2.3–5.6)
Control arm	2.3 (1.8–3.4)	2.2 (1.8–3.1)	2.3 (1.8–3.2)	2.8 (1.8–3.3)

Data are medians (interquartile range). *P < 0.01 vs. healthy volunteers; †P < 0.05 vs. healthy volunteers; ‡P < 0.01 vs. R+ and C–; §P < 0.04 vs. R+, C–, and healthy volunteers.

Antwerp, Belgium) (CV 7%; normal range 1.3–10.4 ng/ml). Plasma levels of vWF antigen were determined by an enzyme-linked immunosorbent assay (ELISA) using rabbit anti-human vWF (Dako A/S, Denmark; CV 7%; normal range 60–180%). TFPI activity was measured using the chromogenic assay according to Sandset et al. (24) and was expressed as a percentage relative to standardized TFPI activity (% normal) measured in a plasma pool obtained from 45 healthy donors (CV 7.4% at the 100% level). ET-1 samples were drawn in EDTA-cooled (0°C) vials and centrifuged at 2,000*g* for 15 min at 4°C. ET-1 was assessed with an endothelin-1 high-sensitivity Biotrak ELISA method (Amersham, Utrecht, the Netherlands) at 450 nm after a Sep-pak C18 column separation.

Serum total cholesterol, HDL cholesterol, and triglyceride levels were determined with enzymatic methods on a Cobas MIRA analyzer; LDL cholesterol levels were calculated using the Friedewald formula; HbA_{1c} and plasma noradrenaline were determined using high-performance liquid chromatography. UAE was measured in two 24-h collections by a commercial immunoturbidimetric method. Samples were stored at –70°C until further processing.

Statistical analysis. Data were analyzed with the Statistical Package for Social Sciences (SPSS/PC Statistical Data Analysis, SPSS, Chicago). All group data are expressed as median values and interquartile ranges, unless otherwise indicated, and nonparametric tests were used for analysis. Between-group data were compared according to Kruskal-Wallis and Mann-Whitney *U* tests. Within-group data were compared according to Friedman's and paired Wilcoxon's signed-rank tests. When more than two comparisons were made, Shaffer-adjusted *P* values were used (25). Correlations between the hemodynamic and circulating markers of endothelial function were explored using only the responses to 30 µg/min ACh (ACh30) and 8 µmol/min L-NMMA (L-NMMA8), because these doses generate maximal responses (26). To find possible determinants for the various aspects of endothelial function, (e.g., L-NMMA8, ACh30, ET-1, PAI-1 activity, t-PA antigen, vWF antigen, and TFPI activity), all these factors were correlated univariately (Pearson's) with age, BMI, smoking habits (cigarettes per day), HDL cholesterol, LDL cholesterol, triglycerides, HbA_{1c}, mean arterial pressure (MAP), and the logarithmic transformed UAE. The presence of an association between the endothe-

TABLE 2
Responses of FBF

	Healthy volunteers	Type 1 diabetic subjects			
		All	C–	R+	MA+
n	12	34	11	10	13
Sodium nitroprusside (µg/min)					
1	34 (16–62)	43 (18–64)	43 (18–57)	60 (24–99)	34 (16–66)
3	182 (145–217)	227 (157–281)	209 (121–241)	268 (135–343)	248 (172–279)
10	286 (208–417)	305 (179–476)	305 (167–405)	444 (154–615)	300 (183–483)
ACh (µg/min)					
3	23 (9–52)	30 (5–48)	12 (3–40)	29 (–2 to 37)	41 (10–138)
10	100 (32–150)	117 (59–237)	119 (40–145)	80 (22 to 205)	157 (67–489)
30	158 (75–217)	173 (102–391)	126 (91–253)	279 (93 to 387)	193 (151–424)
L-arginine +ACh (mg/min + µg/min)					
10 + 3	31 (16–57)	33 (14–68)	33 (15–82)	33 (10–71)	29 (10–59)
10 + 10	126 (57–185)	120 (62–203)	108 (48–168)	226 (88–287)†	131 (48–194)
10 + 30	153 (95–218)	140 (79–238)*	96 (63–176)	221 (133–304)	131 (80–194)

Data are medians (interquartile range) and are given as % change of FBF index. *P = 0.04, L-arginine 10 mg/min +ACh 30 µg/min vs. ACh 30 µg/min. †P = 0.05, L-arginine +ACh 10 µg/min vs. ACh 10 µg/min.

lium-dependent hemodynamic variables (i.e., the vascular responses to L-NMMA8 and ACh30) and ET-1, on the one hand, and the endothelium-dependent hemostatic/fibrinolytic variables (PAI-1 activity, t-PA antigen, vWF antigen, TFPI activity) as well as the endothelial barrier parameter logUAE, on the other, was explored by univariate regression analysis.

Multiple regression analyses were performed to explore the possible influence of diabetes and other independent variables on the various endothelium-dependent hemodynamic and hemostatic/fibrinolytic variables. In this model, the presence or absence of diabetes, BMI, HDL cholesterol, LDL cholesterol, triglyceride, MAP, age, sex, smoking, and the lipid-diabetes interaction variables were used as independent variables. In a separate analysis in the diabetic subjects only, the same model was used with logUAE as additional dependent variable, and HbA_{1c} as additional determinant. P values <0.05 were considered statistically significant.

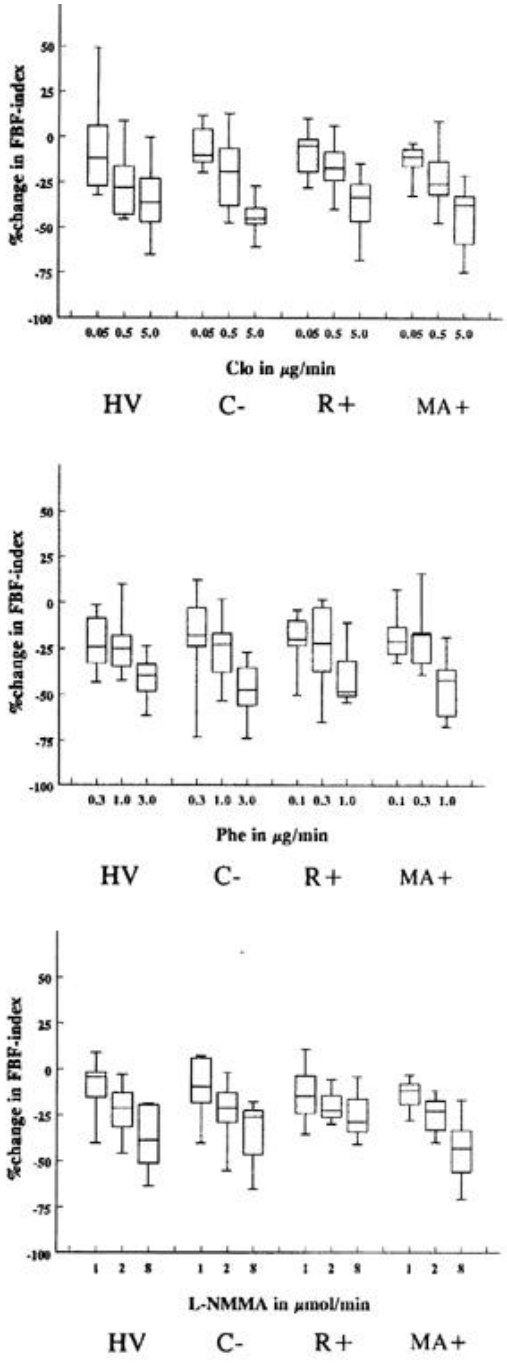

FIG. 1. Vasoconstrictor response of the FBF to clonidine (*A*), to phenylephrine (*B*), and to L-NMMA (*C*). Response of FBF, expressed as percent change in FBF index (FBF infused/FBF control arm). Healthy volunteers (n = 12), C– (n = 11), R+ (n = 10), MA+ (n = 13). Data are expressed as medians, interquartiles (□), and ranges.

RESULTS

Subject characteristics and basal hemodynamic data are given in Table 1. Heart rate was higher in the R+ and MA+ patients, in comparison with the healthy volunteers, and MAP was elevated in the MA+ patients as compared with the C– patients and the healthy volunteers. Sex ratio, age, smoking habits, BMI, lipid profiles, and noradrenaline levels were not different between the diabetic patients and the healthy volunteers. Early morning glucose levels were higher in the diabetic patients. The three diabetic groups had comparable metabolic control. Mean ± SE glucose levels during the experiments were 6.1 ± 0.5 mmol/l in the diabetic subjects, with no differences between the groups. Forearm volume, measured by water volume displacement during submersion of the forearm, was not different between the diabetic subjects and the healthy volunteers (data not shown).

FBF responses to intra-arterial drug infusions. Basal FBF was not different between the groups (Table 1). The FBF response to all vasoconstrictive agents (clonidine, phenylephrine, and L-NMMA) was similar in the diabetic patients and healthy volunteers. Moreover, no differences in vasoconstrictor responses among the three groups of diabetic patients were observed (Fig.1). The FBF response to the vasodilatory drugs ACh and sodium nitroprusside was not different between the diabetic patients and the healthy volunteers, and no differences were observed in this FBF response among the three patient groups either (Table 2).

Administration of exogenous L-arginine (10 mg/min) for 20 min did not influence basal FBF in the forearm of diabetic subjects or the healthy volunteers (data not shown). In the healthy volunteers, the FBF responses to ACh and ACh plus L-arginine were not different (Table 2). In contrast, in the type 1 diabetic patients, the FBF response to the combined infusion of L-arginine and ACh30 μg/min was reduced in comparison with the response to ACh alone: 140 (79–238) versus 173 (102–391)%, P = 0.04 (Table 2). When the different diabetic subgroups were analyzed separately, a similar pattern seemed to be present in C– and MA+ patients (Table 2), while a different pattern was observed in R+ patients. In the latter, the FBF response to combined infusion of L-arginine and ACh, 10 μg/min, was larger than that of ACh alone: 226 (88–287) versus 80 (22–205)%, P = 0.05 (Table 2). Between-group analysis of the diabetic subgroups and healthy volunteers revealed no difference in the ACh response after exogenous L-arginine administration (Table 2). Blood pressure and heart rate did not change during and after the infusion of the various drugs (data not shown).

Endothelial plasma markers. Circulating ET-1 levels were not different between subjects with type 1 diabetes and healthy volunteers (Table 3). In contrast, PAI-1 activity was reduced in the diabetic patients in comparison with healthy volunteers: 6.0 (4.0–8.0) versus 10 (6.3–11.8) ng/ml, P < 0.01. TFPI activity was elevated in the patients with type 1 diabetes as compared with healthy volunteers: 116 (108–125) versus 102 (97–108)%normal, P < 0.001. However, the other endothelial hemostatic/fibrinolytic variables were not different between the diabetic subjects and the healthy volunteers (Table 3).

When the different diabetic subgroups and the healthy volunteers were compared, PAI-1 activity was significantly reduced (P < 0.01) in retinopathic patients (R+) in comparison with other diabetic subgroups and to healthy volunteers (Table 3). Subgroup analysis of the t-PA antigen showed a significant

TABLE 3
Endothelial plasma markers

	Healthy volunteers	Type 1 diabetic subjects		
		C–	R+	MA+
PAI-1 activity (U/ml)	10.0 (6.3–11.8)	7.0 (5.0–8.0)	4.0 (3.0–4.5)*	7.0 (5.5–8.5)
t-PA antigen (ng/ml)	5.2 (3.1–6.5)	2.4 (1.5–3.0)†	4.4 (2.9–6.1)	3.7 (2.7–5.4)
vWF antigen (% normal)	106 (76–158)	87 (70–145)	93 (84–136)	100 (83–112)
TFPI activity (% normal)	102 (97–108)	113 (108–125)†	109 (105–122)‡	118 (114–127)†
ET-1 (fmol/l)	0.88 (0.76–1.14)	1.49 (0.67–2.79)	0.72 (0.64–0.84)	0.69 (0.55–1.30)

Data are medians (interquartile range). *P < 0.01 vs. healthy volunteers, C–, MA+; †P < 0.05 vs. healthy volunteers; ‡P < 0.06 vs. healthy volunteers.

reduction in C– subjects in comparison with healthy volunteers (Table 3). The TFPI activity was elevated in C– and MA+ subjects in comparison with healthy volunteers (Table 3).

Univariate regression analyses. Univariate analysis showed that ACh30 and L-NMMA8 in all subjects, and logUAE in the patients with type 1 diabetes, were positively related to MAP (Table 4). TFPI activity was inversely related to age in all subjects (r = –0.41, P < 0.01). In the diabetic subjects, TFPI was inversely related to age (r = –0.43, P < 0.05) and HDL cholesterol (r = –0.39, P < 0.05), and positively to triglyceride (r = 0.43, P < 0.05). vWF antigen was inversely related to smoking habits (r = –0.39, P < 0.05) in these patients. No other significant correlations were found between endothelium-dependent factors and possible explanatory variables.

As a second step, endothelium-dependent vasomotor responses, hemostatic/fibrinolytic factors, and logUAE were correlated to explore the presence of synchronization of changes in these various parameters (Table 4). This analysis revealed that endothelium-dependent vasodilatation to ACh30 was inversely related to PAI-1 activity (r = –0.37, P < 0.05). Furthermore, PAI-1 activity was positively correlated with logUAE in the type 1 diabetic patients (r = 0.48, P < 0.01).

Multivariate analyses. Multivariate analyses were performed to explore the influence of diabetes on endothelium-dependent factors, correcting for possible interaction of various subject characteristics. After multiple linear regression analysis, TFPI activity remained the only endothelium-dependent variable that was significantly related to the presence of diabetes (β = 0.52, P < 0.001). TFPI was further dependent on BMI (β = 0.38, P < 0.01), age (β = –0.40, P = 0.001), and LDL cholesterol (β = 0.32, P < 0.01). All other endothelium-dependent variables were not influenced by the presence of diabetes. ACh30 was related to MAP (β = 0.34, P < 0.05) and BMI (β = –0.30, P < 0.05). L-NMMA8 was only related to MAP (β = 0.41, P < 0.01). In the diabetic subjects, logUAE was related to MAP (β = 0.46, P < 0.01). Furthermore, vWF levels were determined by active smoking (number of cigarettes; β = –0.40, P < 0.05) and HDL cholesterol (β = –0.38, P < 0.05) in the subjects with type 1 diabetes. The other endothelium-dependent hemostatic/fibrinolytic variables were not dependent on the explored subject characteristics.

DISCUSSION

Endothelium-derived NO and endothelial plasma markers. The present study clearly demonstrates that under near-normoglycemic conditions, both basal and stimulated endothelium-dependent vasodilatation of the forearm are intact in type 1 diabetic patients with or without microvascular complications, compatible with normal endothelium-derived NO availability. However, repeated stimulated endothelium-dependent vasodilatation in the presence of exogenous L-arginine resulted in a reduced response. TFPI activity was the only plasma marker that was elevated consistently in type 1 diabetic patients. In our opinion, the present data reject the current paradigm of a generalized endothelial dysfunction in type 1 diabetes, in which one endothelium-dependent variable can be used as a marker of or a substitute for alterations in different aspects of endothelial function.

Animal studies clearly suggest that endothelium-dependent vasodilatation is altered in experimental diabetes, which is probably based on altered production or degradation of NO (27). Human studies in type 1 diabetes are less consistent. These studies seem difficult to reconcile with several human studies, which, however, also have produced some conflicting results (4–11,28). However, when various studies are combined, a clearer picture seems to emerge. In the majority of studies, basal and stimulated endothelium-dependent vasodilatation were normal in the forearm of patients without diabetic complications and normal cholesterol levels, as also shown in the present study (3–8). In contrast, in the femoral artery, Zenere et al. (9) observed a reduction in both endothelium-dependent and endothelium-independent vasodilatation in a relatively small number of patients without microvascular complications, who were studied postprandially (blood glucose levels were not reported). These data suggest that in the femoral artery the sensitivity to NO be reduced in type 1 diabetic patients. Also, in type 2 diabetic patients, we observed in an earlier study a reduced endothelium-independent vasodilatation of the femoral artery (29), and further studies are needed on the possible regional differences in NO sensitivity in diabetes.

Animal studies also suggest that severe metabolic decompensation, with glucose levels >20 mmol/l and insulin deficiency, impairs endothelium-dependent vasodilatation. Low-

TABLE 4
Univariate correlations

	MAP	Age	PAI-1
ACh30	0.31*	NS	–0.37*
L-NMMA8	0.41†	NS	NS
LogUAE (type 1)	0.53†	NS	0.48†
TFPI activity	NS	–0.41†	NS

Data are r. *P < 0.05; †P < 0.01.

ering of blood glucose levels to values more relevant for the diabetic patient by insulin therapy restores this impaired vasodilatation (30). Hyperglycemia, per se (up to 15 mmol/l), does not affect endothelium-dependent vasodilatation in healthy volunteers (22). Insulin, however, augments both basal and stimulated endothelium-dependent vasodilatation in skeletal muscle (31). On the basis of these data, we conclude that in patients with type 1 diabetes (chronic), moderate hyperglycemia in itself does not affect endothelium-dependent vasodilatation but, when these patients become insulin deficient, NO synthesis and/or release probably decreases. Indeed, Johnstone et al. (7) found in poorly controlled type 1 diabetic patients (mean blood glucose 14 mmol/l) a reduced metacholine stimulated forearm vasodilatation, which correlated inversely with the serum insulin concentration ($r = -0.60$).

In patients with atherosclerosis, hyperlipidemia, or type 2 diabetes, intra-arterial L-arginine (but not D-arginine) administration augments ACh-mediated vasodilatation (32–34). Also in some, but not all, studies in diabetic animals, an improvement of endothelium-dependent vasodilatation was observed after L-arginine supplementation (14–16,35). In our type 1 diabetic patients without microangiopathy or with microalbuminuria, we did not find any evidence that L-arginine improves endothelium-dependent vasodilatation. In contrast, the maximal response to the second ACh infusion was reduced in comparison to the first ACh infusion, despite the presence of exogenous L-arginine. No such changes were observed in healthy volunteers, which is in accordance with earlier studies in healthy Caucasian subjects (32). At present, this decreased response is difficult to explain but could be based on factors such as receptor desensibilization, intracellular substrate depletion, or second messenger depletion. Whether reduced endothelium-dependent vasodilatation after repeated stimulation can also be observed with other stimuli of NO synthesis needs to be studied. In contrast to the aforementioned patient groups, the patients with retinopathy had an enhanced vasodilator response during repeated ACh infusion and L-arginine coinfusion, which could suggest that in only these patients L-arginine can improve endothelium-dependent vasodilatation. Pieper et al. (14) observed that L-arginine can improve ACh-mediated vasodilatation in rats after 8 weeks, but not after 12 weeks of diabetes. These data suggest that depending on the duration of the disease, a reversible or irreversible defect was present. However, differences in species must also be taken into account as no improvement after L-arginine was observed in diabetic hamsters with 2 weeks of diabetes (16). In our study the duration of the disease did not differ between the patient groups, nor was it correlated with ACh-mediated vasodilatation. It should be noted that the subgroup with retinopathy consisted of a relatively small number of patients and the data therefore should be interpreted with caution, because a type 2 error cannot be excluded. Clearly, further studies are necessary on the nature of the enhancement of endothelium-dependent vasodilatation by L-arginine in patients with retinopathy.

Diabetic nephropathy is closely associated with multiple abnormalities in both micro- and macrocirculation and is a marker of generalized vascular disease (1). In separate studies, disturbances in endothelial vasomotor, hemostasis/fibrinolysis, and barrier function have been observed in patients with (incipient) nephropathy, suggesting that endothelium-derived NO production could also be impaired (1). However, we found normal endothelium-dependent vasodilatation in the forearm of microalbuminuric patients. Our data are in accordance with other recent studies in nondiabetic hypertensive subjects with microalbuminuria and in type 1 diabetic subjects with overt nephropathy (8,36). In contrast to our study, Elliot et al. (5) reported a reduction in the vasoconstrictor response to L-NMMA in microalbuminuric patients with characteristics very similar to ours. Possibly, differences in experimental protocol could explain this discrepancy. In our protocol, L-NMMA and ACh were infused on different days. In the study of Elliot et al. (5) carbachol (like ACh, a muscarinergic agonist) and L-NMMA were infused with washout periods of only 6–15 min. This must have resulted in carryover effects, which hamper any comparison of the two studies (37).

Studies on endothelial function in diabetes are usually limited to certain aspects (e.g., hemostatic or vasomotor function), which are then used as marker(s) of generalized endothelial damage or endothelial dysfunction. Of the several endothelium-dependent variables determined in the present study, only TFPI activity and PAI-1 activity were significantly different between patients with type 1 diabetes and healthy subjects. TFPI activity was most markedly elevated in the microalbuminuric patients and PAI-1 activity was correlated with UAE in the diabetic patients, thus confirming earlier studies (17,18). After synthesis by the vascular endothelium, TFPI is bound to the endothelial surface and to a lesser degree released into plasma (38). In plasma TFPI is mainly transported by lipoproteins (39), which probably explains the association between TFPI and LDL cholesterol in the present study. A new observation was the inverse correlation between PAI-1 activity and ACh stimulated endothelium-dependent vasodilatation. As NO inhibits platelet aggregation and adhesion to the endothelium, a simultaneous rise in PAI-1 activity and decrease in NO synthesis and/or release would favor the formation of thrombi at the endothelial surface. Subgroup and univariate analysis clearly suggested that no other consistent pattern of multiple endothelial abnormalities could be observed in our diabetic patients.

Adrenergic responses. Human studies on vascular adrenergic responsiveness in type 1 diabetes are, to our knowledge, scarce. The normal adrenergic responsiveness of forearm arterioles in our patients is in accordance with an earlier study in type 1 diabetic patients without complications by Halkin et al. (3). In an earlier study, we found that in type 1 diabetic patients with microalbuminuria, the venous constrictor responses to clonidine are enhanced, which is probably based on a venous increased α_2 adrenoceptor activity (21). In contrast, the constrictor responses of forearm arterioles to clonidine were unaltered in our microalbuminuric patients. When this increased α_2 adrenergic responsiveness, indeed, is limited to the postcapillary vascular bed (in particular, the venules), it could result in a rise in postcapillary resistance. Subsequently, this rise in postcapillary resistance could be the basis of the decrease in skin-blood flow and an elevation of intracapillary blood pressure in microalbuminuric patients, as observed in separate studies (40,41). Further studies are necessary on the mechanism(s) and consequences of the different adrenergic responsiveness of peripheral veins and arterioles in microalbuminuric patients.

Limitations of the study. Endothelial function is affected by many factors, whereas the principal aim of our study was to determine the effect of long-term diabetes and its complications on endothelial function and adrenergic responsiveness. Therefore, the present data cannot be extrapolated to type 1 diabetic patients with poor metabolic control, hyperlipidemia, or hypertension. A further limitation is that vascular responses were studied in forearm vessels only. It remains to be determined whether our conclusions can also be applied to the eye or the kidney, but a close association between endothelium-dependent vasodilatation in the forearm and the coronary vessels has been observed (42). Apart from the technique, the organ, and the vascular bed studied, the results are also affected by the type of drug, which is used to block or stimulate NO production. We assessed stimulated endothelium-dependent vasodilatation indirectly by infusing ACh, which might also affect vascular prostanoid and endothelium-derived hyperpolarizing factor (EDHF) production. ACh mediated vasodilatation in the forearm, very likely, is not mediated by prostanoids (43). However, based on the present data, we cannot exclude that EDHF production is altered in type 1 diabetes. A further limitation of our study was that control experiments with infusion of D-arginine (instead of L-arginine) were not performed. Finally, it should be noted that the present study did not address the effect of (autonomic) neuropathy. In patients with autonomic neuropathy, adrenergic vascular responses are increased, and loss of autonomic function in patients with type 1 diabetes is associated with enhanced endothelium-independent vasodilatation in the forearm, as recently reported by Mäkimattilla (8).

In conclusion, forearm adrenergic responsiveness, as well as endothelium-dependent vasodilatation seem unaltered in type 1 diabetic patients with and without microvascular complications during normoglycemic and near-normoglycemic conditions. However, in contrast to healthy control subjects vasodilatation is depressed during repeated ACh stimulation (during concomitant L-arginine administration) in patients without complications or with microalbuminuria. Further studies are needed to determine if this decrease in vasodilatation is based on an early exhaustion of or desensibilization to endothelium-derived NO. Alternatively, this decreased vasodilatation could be based on changes in the release of other vasoactive agents by the endothelium. In contrast, in patients with retinopathy, endothelium-dependent vasodilatation during L-arginine supplementation is enhanced. Finally, no consistent pattern of generalized endothelial dysfunction (or damage) is observed in type 1 diabetic patients with or without microvascular complications. Elevation of TFPI was the most consistent marker of (early) endothelial damage or activation. Prospective studies are needed to determine whether TFPI can be used as a marker for the development of microangiopathic complications in type 1 diabetes.

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