Soluble Intercellular Adhesion Molecule, Vascular Cell Adhesion Molecule, and Impaired Microvascular Reactivity Are Early Markers of Vasculopathy in Type 2 Diabetic Individuals Without Microalbuminuria

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OBJECTIVE — Using von Willebrand Factor (vWF) as a marker of endothelial function, previous studies have shown that the development of microalbuminuria is associated with the onset of endothelial dysfunction in individuals with type 2 diabetes. We tested the hypothesis that endothelial dysfunction is already evident in normoalbuminuric individuals with type 2 diabetes.

RESEARCH DESIGN AND METHODS — We used laser Doppler imaging scanning to measure vasodilation in the forearm skin in response to iontophoresis of 1% acetylcholine (endothelium-dependent) and 1% sodium nitroprusside (endothelium-independent). Multiple indicators of endothelial function—soluble intercellular adhesion molecule (sICAM), soluble vascular cell adhesion molecule (sVCAM), vWF, and microvascular reactivity—were measured in 20 healthy control subjects, 45 normoalbuminuric (urinary albumin/creatinine ratio <30 μg/mg) individuals with type 2 diabetes, and 14 microalbuminuric (urinary albumin/creatinine ratio between 30 and 300 μg/mg) individuals with type 2 diabetes.

RESULTS — Serum sICAM and sVCAM levels were elevated in the normoalbuminuric (305 ± 120, 851 ± 284 ng/ml) and microalbuminuric (300 ± 89, 845 ± 252 ng/ml) individuals with diabetes when compared with the healthy control subjects (213 ± 58, 661 ± 176 ng/ml) ($P < 0.01$). Furthermore, the microvascular endothelium-dependent and -independent vasodilation was reduced in the normoalbuminuric (76 ± 44, 70 ± 33) (percent increase in perfusion over baseline) and microalbuminuric (74 ± 41, 73 ± 28) individuals with diabetes compared with healthy control subjects (126 ± 67, 120 ± 47) ($P < 0.05$). In contrast, plasma vWF was elevated only in the microalbuminuric individuals with diabetes (129 ± 35%) compared with the normoalbuminuric individuals with diabetes (110 ± 34) and healthy control subjects (111.3 ± 39) ($P < 0.05$). On stepwise multivariate analysis, fasting blood glucose was the most important contributing factor to the variation in microvascular reactivity and sVCAM, whereas insulin resistance (by homeostasis model assessment) was the most important contributing factor to the variation in sICAM. Addition of all clinical and biochemical measures explained only 15–22% of the variation in sICAM, sVCAM, and microvascular reactivity.

CONCLUSIONS — Multiple markers of endothelial dysfunction were evident in normoalbuminuric individuals with type 2 diabetes. The pathogenic process of vasculopathy in type 2 diabetes occurs early and may be operative before the development of microalbuminuria.

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In individuals with type 1 or type 2 diabetes, microalbuminuria predicts not only the progression of nephropathy but also cardiovascular morbidity and mortality (1–3). In addition, it has been shown that microalbuminuria is also a predictor of vascular disease in nondiabetic subjects (4). The mechanism(s) underlying this association are unclear. It has been proposed that the presence of microalbuminuria may reflect a generalized defect in vascular permeability and a concomitant atherogenic diathesis (i.e., endothelial dysfunction) (5). Furthermore, microalbuminuria has been found to be closely associated with insulin resistance in hypertensive individuals and subjects with diabetes (6,7).

The plasma concentration of vWF has been used as an indicator of endothelial dysfunction and has been shown to increase in subjects with type 2 diabetes concomitant with the development of microalbuminuria. In contrast, vWF levels did not rise in those individuals with type 2 diabetes who remained normoalbuminuric throughout the period of follow-up (8). These results suggest that endothelial dysfunction occurs in parallel with the development of microalbuminuria. On the other hand, in another study that included type 1 diabetic patients, the rise of vWF preceded the development of microalbuminuria (9). In the present study, we have compared the microvascular reactivity and plasma levels of various markers of endothelial activation in type 2 diabetic

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Abbreviations: ACh, acetylcholine; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; HOMA, homeostasis model assessment; ICAM, intercellular adhesion molecule; IR, insulin resistance; OGTT, oral glucose tolerance test; s, soluble; SNP, sodium nitroprusside; VCAM, vascular cell adhesion molecule; VSMC, vascular smooth muscle cell; vWF, von Willebrand Factor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

patients with and without microalbuminuria with those of healthy control subjects.

RESEARCH DESIGN AND METHODS

Study subjects

We studied a total of 78 subjects, age 33–69 years, who were divided into three groups. The first group consisted of 20 healthy subjects, the second of 45 patients with type 2 diabetes without microalbuminuria, and the third group of 14 patients with type 2 diabetes with microalbuminuria. Normoalbuminuria was defined as spot urine albumin to creatinine ratio <30 μg/mg, whereas microalbuminuria was defined as spot urine albumin to creatinine ratio between 30 and 300 μg/mg. All the healthy subjects had a normal oral glucose tolerance test (OGTT) before entry into the study. The diabetic individuals had the diagnosis of type 2 diabetes established before they were screened for the study and did not require insulin therapy for glucose control. Diabetes and normal glucose tolerance were defined according to the recommendations of the ADA Expert Committee on the Classification and Diagnosis of Diabetes (10).

To avoid confounding factors known to affect endothelial function and/or glucose metabolism, the following exclusion criteria were enforced: smoking any amount of cigarettes during the previous 6 months; subjects with past history of cardiovascular disease (coronary artery disease, arrhythmia, heart failure), stroke, or transient ischemic attack; peripheral vascular disease; chronic renal disease (serum creatinine >1.5 mg/dl); severe dyslipidemia (serum triglycerides >600 mg/dl or cholesterol >300 mg/dl); or any other serious chronic disease requiring active treatment. Subjects were also excluded if they were on any of the following medications: any type of antihypertensives, lipid-lowering agents, glucocorticoids, antineoplastic agents, psychoactive agents, or bronchodilators. In addition, diabetic patients with proliferative retinopathy, peripheral somatic neuropathy, and/or macroalbuminuria (expressed as albumin/creatinine ratio, >300 μg/mg) were also excluded.

Procedures

Subjects were studied after an overnight fast and a 24-h period of abstinence from alcohol and vigorous exercise. A standard 75-g OGTT was performed in those individuals without known history of diabetes to evaluate their glucose status. Eligible individuals were asked to come back for a second visit to the Joslin Clinical Research Center after an overnight fast of 12 h. A general physical examination was performed by a study physician. The diagnosis of proliferative retinopathy was made on the basis of clinical examination or a history of previous retinal laser treatment. The systolic and diastolic blood pressure readings were recorded to the nearest 2 mmHg as the mean of two measurements with the subjects seated. Subjects' weight, height, and waist-to-hip ratio were also obtained. The BMI was calculated by dividing the weight in kilograms by the square of the height in meters.

Blood samples were drawn from an antecubital vein with a 19-gauge needle without venous stasis. Plasma glucose, total serum cholesterol, and triglycerides were measured using the Synchron CX analyzer (Beckman Systems, Brea, CA), whereas HDL serum cholesterol was measured directly (Sigma, St. Louis, MO). LDL cholesterol levels were calculated using the Friedwald formula. HbA_{1c} (normal range 4–6%) was determined in whole blood using ion-exchange high-performance liquid chromatography (HPLC). Plasma insulin was measured using the radioimmunoassay method. The enzyme-linked immunosorbent assay (ELISA) method was used to measure the soluble intercellular adhesion molecule (sICAM), the soluble vascular cell adhesion molecule (sVCAM) (R&D Systems Minneapolis, MN), and vWF (American Bioproducts, Parsippany, NJ). The sICAM and sVCAM assay had an intra-assay coefficient of variation (CV) of 4.8 and 5.9%, respectively, and an interassay CV of 10.1 and 10.2%, respectively. The vWF ELISA system had an intra-assay CV of 6.3% and an interassay CV of 7.2%. Relative insulin resistance (IR) was calculated using homeostasis model assessment (HOMA) (11).

Assessment of vascular reactivity

All vascular reactivity measurements were performed on the same morning as the clinical evaluation while the subjects were still fasting. The investigators who performed the measurements (S.C.L., P.S.) were blinded to the medical history of the subjects.

The skin over the extensor surface of the forearm was tested by performing laser Doppler perfusion imaging measurements before and after the iontophoresis of ACh and sodium nitroprusside (SNP). Iontophoresis is a noninvasive technique that avoids any systemic effects of the used drugs. Acetylcholine (ACh) chloride was used to assess endothelium-dependent vasodilation because its main effect is to stimulate endothelial cell production and/or release of nitric oxide (NO). SNP was used to assess endothelium-independent vasodilation because it directly relaxes vascular smooth muscle cells (VSMCs), "bypassing" endothelial cell generation of NO. We used the MIC1 iontophoresis system (Moor Instruments, Millwey, Devon, U.K.). The iontophoresis chamber was filled with a small quantity (<1 ml) of 1% ACh chloride solution or SNP, and a constant current of 200 μA was then applied for 60 s, achieving a dose of 6 mC/cm^2. The changes in the superficial cutaneous blood vessel perfusion were assessed before and after the iontophoresis by a Laser Doppler Perfusion Imager (Lisca PIM 1.0, Lisca Development, Linkoping, Sweden). The Imager uses a 1-MW helium-neon laser beam of 633-nm wavelength that sequentially scans the skin area where iontophoresis is performed. The maximum number of measured spots is 4,096, and the apparatus produces a color-coded image of skin erythrocyte flux on a computer monitor. The reproducibility of the technique has been previously reported by our group (12). The CV was 14.1% for the baseline measurement and 13.7% during maximal hyperemic response after the iontophoresis.

The protocol was approved by the institutional review board at each center, and all participants gave written informed consent. Volunteers for the study were recruited through local advertisement at the Joslin Diabetes Center and The Beth Israel Deaconess Medical Center in Boston.

Data analysis

The Minitab statistical package version 12.0 (Minitab, State College, PA) for personal computers was used for the statistical analysis. Comparisons of clinical and biochemical parameters and microvascular reactivity among the three groups of individuals were analyzed using one-way analysis of variance followed by the Fisher's test in order to identify differences among groups. Parameters that were not normally distributed were compared using the Kruskal-Wallis test. Proportion of sex differences among the groups were examined using the χ^2 test. Correlation between variables was tested using Pearson correlation analysis. Stepwise regression analysis was

Table 1—*Clinical and biochemical characteristics of subjects studied*

	Control subjects (group C)	Diabetic normoalbuminuric subjects (group D)	Diabetic microalbuminuric subjects (group DA)
n	20	45	14
Age (years)	53 ± 6	54 ± 10	55 ± 9
Sex (M/F)	10/10	14/31	5/9
Duration of diabetes (years)	—	5.1 ± 5.8	5.4 ± 3.7
BMI (kg/m^2)	27 ± 4*	32 ± 6	34 ± 4
Systolic blood pressure (mmHg)	113 ± 11†	126 ± 12	128 ± 7
Diastolic blood pressure (mmHg)	73 ± 9‡	80 ± 6	81 ± 6
Albumin/creatinine (μg/mg)	3.3 ± 1.5	5.7 ± 4.2	76 ± 73§
Fasting glucose (mg/dl)	92 ± 8‖	147 ± 30	226 ± 61
Relative insulin resistance (HOMA model)	1.3 ± 0.8¶	6.6 ± 5.5	10.2 ± 5.6
HbA_{1c} (%)	5.6 ± 0.5#	7.4 ± 0.8	9.4 ± 1.7
Insulin (μU/ml)	5.6 ± 3**	18 ± 15	18 ± 9
Total cholesterol (mg/dl)	196 ± 29††	205 ± 34	233 ± 33
HDL (mg/dl)	52 ± 16‡‡	43 ± 11	46 ± 12
LDL (mg/dl)	122 ± 27§§	124 ± 32	156 ± 39
Triglyceride (mg/dl)	99 ± 47‖‖	197 ± 95	213 ± 103

Data are means ± SD. *C vs. D and DA, $P < 0.001$; †C vs. D and DA, $P < 0.001$; ‡C vs. D and DA, $P < 0.001$; §C and D vs. DA, $P < 0.001$; ‖C vs. D vs. DA, $P < 0.001$; ¶C vs. D vs. DA, $P < 0.001$; #C vs. D vs. DA, $P < 0.001$; **C vs. D and DA, $P < 0.001$; ††C and D vs. DA, $P < 0.001$; ‡‡C vs. D, $P < 0.001$; §§C and D vs. DA, $P < 0.001$; ‖‖C vs. D and DA, $P < 0.001$.

performed to determine the relationship between the markers of endothelial function and the following parameters: BMI, IR, systolic and diastolic blood pressure, fasting blood glucose, fasting insulin, and HbA_{1c}. A *P* value of ≤0.05 was considered statistically significant.

RESULTS — The clinical characteristics and biochemical profile of the subjects are shown in Table 1. The subjects' age and sex were comparable among the groups. Duration of diabetes was similar between the normoalbuminuric and microalbuminuric diabetic subjects. BMI was higher in the individuals with diabetes compared with the healthy control subjects ($P < 0.001$). Systolic and diastolic blood pressures were also higher in the subjects with diabetes compared with the healthy control subjects ($P < 0.001$).

The ratio of urinary albumin to creatinine was higher in the microalbuminuric subjects with diabetes compared with the normoalbuminuric subjects with diabetes and healthy control subjects ($P < 0.001$). Fasting blood glucose and HbA_{1c} were higher in the microalbuminuric subjects with diabetes compared with the normoalbuminuric subjects with diabetes and healthy control subjects ($P < 0.001$). They were also higher in the normoalbuminuric subjects with diabetes compared with the healthy control subjects ($P < 0.001$). Fasting insulin levels were higher in the subjects with diabetes compared with the healthy control subjects ($P < 0.001$).

Total cholesterol levels were higher in the microalbuminuric subjects with diabetes compared with the normoalbuminuric subjects with diabetes and healthy control subjects ($P < 0.01$). HDL was lower in the normoalbuminuric subjects with diabetes compared with the healthy control subjects ($P < 0.05$). LDL was higher in the microalbuminuric subjects with diabetes compared with the normoalbuminuric subjects with diabetes and healthy control subjects ($P < 0.01$). Triglyceride levels were higher in the subjects with diabetes compared with the healthy control subjects ($P < 0.001$). Plasma sICAM and sVCAM levels were higher in the subjects with diabetes compared with the healthy control subjects ($P < 0.01$). Plasma vWF was higher in the microalbuminuric subjects with diabetes compared with the normoalbuminuric subjects with diabetes and healthy control subjects ($P < 0.05$).

The results of microvascular reactivity are shown in Table 2 and Fig. 1. The percentage increase in perfusion over baseline after the iontophoresis of ACh and SNP was reduced in the subjects with diabetes compared with the healthy control subjects ($P < 0.05$ and $P < 0.001$, respectively).

When all subjects were considered as one group, correlation analysis showed a significant correlation between sICAM and sVCAM ($r = 0.72$, $P < 0.001$). There was also a significant correlation between the response to ACh and SNP ($r = 0.49$, $P < 0.001$). There were significant inverse correlations between sICAM, sVCAM, and response to SNP ($r = 0.35$, $P < 0.01$ and $r = 0.32$, $P < 0.05$, respectively). The correlations between sICAM, sVCAM, and response to ACh failed to reach statistical significance.

Table 3 shows the relationship between sICAM, sVCAM, and microvascular reactivity and various clinical and metabolic parameters. A significant correlation was found between the response to ACh, SNP, and sICAM levels and IR, fasting insulin, fasting blood glucose, and HbA_{1c}. sVCAM was significantly correlated with fasting

Table 2—*Markers of vascular function in subjects studied*

	Control subjects (group C)	Diabetic normoalbuminuric subjects (group D)	Diabetic microalbuminuric subjects (group DA)
sICAM (ng/ml)	213 ± 58*	305 ± 120	300 ± 89
sVCAM (ng/ml)	654 ± 173†	839 ± 238	795 ± 297
vWF (%)	111 ± 39‡	110 ± 34	154 ± 35
Mean skin temperature (°C)	31.0 ± 0.7	30.7 ± 0.6	30.8 ± 0.7
Percentage increase in perfusion over baseline after ACh iontophoresis	110 ± 44§	75 ± 43	74 ± 41
Percentage increase in perfusion over baseline after SNP iontophoresis	122 ± 41‖	73 ± 35	73 ± 28

Data are means ± SD. *C vs. D and DA, $P < 0.05$; †C vs. D and DA, $P < 0.05$; ‡C and D vs. DA, $P < 0.001$; §C vs. D and DA, $P < 0.05$; ‖C vs. D and DA, $P < 0.001$.

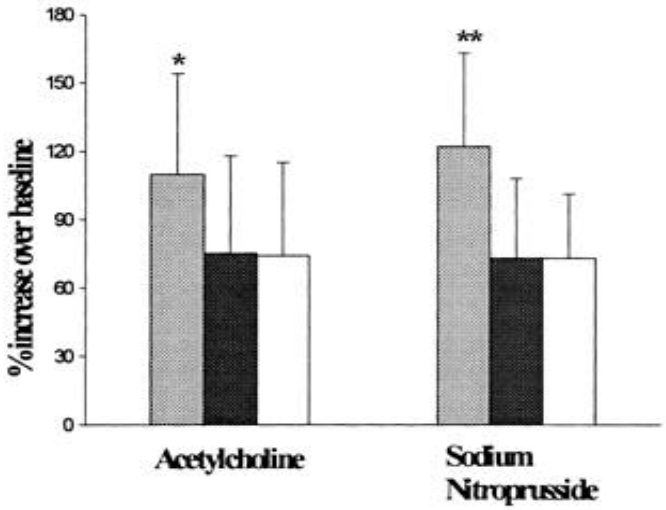

Figure 1—*The results of iontophoresis of ACh (endothelium-dependent vasodilation) and SNP (endothelium-independent vasodilation) on the forearm skin in healthy subjects (■), normoalbuminuric (■) and microalbuminuric (□) subjects with type 2 diabetes. The responses to ACh ($P < 0.05$) and SNP ($P < 0.001$) were reduced in the normoalbuminuric and microalbuminuric subjects with type 2 diabetes compared with the healthy controls. *$P < 0.05$, **$P < 0.001$.*

blood glucose and HbA_{1c}. Stepwise regression revealed that only IR significantly contributed to 15.8% of the variation of sICAM, whereas fasting blood glucose contributed to 9.8% of variation in sVCAM. Fasting blood glucose was the only statistically significant contributing factor to the response to ACh and SNP and accounted for 11.0 and 14.3% of the variation, respectively. The addition of all the following factors in the model—BMI, IR, systolic and diastolic blood pressure, fasting blood glucose, fasting insulin, and HbA_{1c}—increased the prediction of variation in sICAM and sVCAM and the response to ACh and SNP to 22.0, 16.0, 15.1, and 19.9%, respectively.

CONCLUSIONS — The main finding of this study was the presence of markers of endothelial dysfunction (elevations of sICAM and sVCAM but not of vWF) and impaired endothelium-dependent vasodilation in normoalbuminuric subjects with type 2 diabetes. Previously, microalbuminuria has been considered to be a surrogate marker of endothelial dysfunction, thereby providing the link between abnormal albumin excretion and excessive cardiovascular morbidity and mortality. However, our data demonstrate that endothelial function is already impaired in normoalbuminuric subjects with type 2 diabetes.

It is now increasingly recognized that the endothelial-leukocyte adhesion molecules play an important role in the physiological adaptation and pathophysiological dysfunction of the vasculature. A recent large-scale nested case-control study showed that baseline plasma sICAM concentration is an independent predictor for future myocardial infarction in apparently healthy men (13). In addition, circulating sVCAM was found to be a marker of atherosclerotic lesions in type 2 diabetic patients with symptomatic and asymptomatic atherosclerosis (14). However, it should be also remembered that ICAM and VCAM are not exclusively expressed by the endothelial cells and that other cell types also express these molecules. Thus, elevated sICAM and sVCAM should be considered as markers of endothelial dysfunction rather than the direct results of such a dysfunction.

The normoalbuminuric subjects with type 2 diabetes in our study had elevation of both the sICAM and sVCAM levels compared with the control subjects. Therefore, although the precise contribution of measurable plasma sICAM and sVCAM by the endothelium has yet to be quantified, our data are consistent with the notion that the normoalbuminuric individuals with type 2 diabetes are already at increased risk for cardiovascular diseases, probably because of endothelial activation. Moreover, recent studies have also shown that increased circulating levels of sVCAM are associated with diabetic retinopathy (15) and nephropathy (16) and that elevated sICAM may also have a role in the pathogenesis of diabetic neuropathy (17). Hence, normoalbuminuric subjects with type 2 diabetes and elevated levels of the adhesion molecules would also be at risk for microvascular complications.

Interestingly, there was a differential activation of the biochemical markers of endothelial function in normoalbuminuric subjects with type 2 diabetes whereby sICAM and sVCAM, but not vWF, were elevated. In other words, endothelial activation may result in the expression of one adhesion molecule but not the others. Evidence from in vitro experiments also suggests that the induction and modulation of adhesion molecule expression in the endothelium is complex and selective (18). Therefore, effective screening for endothe-

Table 3—*Correlations of sICAM, sVCAM, and microvascular reactivity with clinical and metabolic parameters*

	sICAM		sVCAM		Response to ACh		Response to SNP	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Systolic blood pressure	0.196	0.145	0.106	0.441	−0.142	0.213	−0.214	0.059
Diastolic blood pressure	0.026	0.846	−0.003	0.983	−0.188	0.099	−0.108	0.345
BMI	0.122	0.368	0.074	0.594	−0.139	0.227	−0.159	0.167
Insulin resistance (HOMA)	0.404	0.002	0.152	0.269	−0.317	0.005	−0.368	0.001
Fasting insulin	0.322	0.015	0.088	0.522	−0.249	0.030	−0.304	0.008
Fasting blood glucose	0.390	0.003	0.323	0.016	−0.335	0.003	−0.377	0.001
HbA_{1c}	0.374	0.004	0.305	0.023	−0.281	0.013	−0.369	0.001

lial dysfunction in subjects with type 2 diabetes may require the measurement of multiple biochemical markers.

Some differences in clinical and metabolic characteristics were observed among our study groups (Table 1). Not surprisingly, the subjects with diabetes were more obese, were more insulin resistant, and had higher blood pressure (although the readings were well within the normal range). Because these parameters are characteristics of the metabolic syndrome and cannot be considered as independent from the presence of diabetes, we felt that matching the groups for these factors would result in including in the control group subjects who were not healthy but were at risk of developing diabetes. The fact that all these factors have been associated with endothelial dysfunction further corroborates this notion (19,20). Hence, we used correlation analysis and stepwise linear regression analysis to adjust the impact of these variables on the variation in markers of endothelial function in our study. There was significant correlation between sICAM and microvascular reactivity and IR, fasting insulin, fasting blood glucose, and HbA_{1c} (Table 3). There was also significant correlation between sVCAM and fasting glucose and HbA_{1c}. However, during stepwise regression, only fasting glucose was identified as a significant contributor to microvascular reactivity and plasma concentrations of sVCAM, whereas IR was identified as a significant contributor to plasma sICAM. On multiple linear regression, only 15–22% of the variation in sICAM and sVCAM levels and microvascular reactivity can be explained by the addition of all the relevant clinical and metabolic factors. Therefore, IR and blood pressure differences may have accounted for some of the observed variation in endothelial function among the study groups. However, our data would argue that these clinical and metabolic factors were insufficient to explain all the observed differences in endothelial function. Therefore, a combination of genetic factors and acquired metabolic abnormalities may interact to determine vascular health and disease (21).

The decreased responsiveness to the iontophoresis of ACh or SNP in subjects with diabetes is not related to decreased absorption of the applied pharmaceutical stimuli because there is no histological difference in the cutaneous capillary density and skin thickness in the forearm in healthy control subjects and individuals with diabetes (22). Moreover, noninvasive measurements of the skin capillary density in the upper extremity have shown no differences between individuals with diabetes and healthy control subjects (23). Therefore, absorption of pharmacological stimuli should not be limited by the availability of cutaneous vasculature, and a blunted vascular response to ACh and SNP among the subjects with diabetes was most likely the result of impaired vascular reactivity.

We also found impairment of both microvascular endothelium-dependent and -independent vasodilation in normoalbuminuric subjects with type 2 diabetes. Hence, vascular dysfunction in the diabetic patients was not limited to the endothelium but also involved the VSMC. Therefore, it has been suggested that the abnormal response to endogenous and exogenous NO donors implicates either increased inactivation of NO by reactive oxygen species or abnormalities of signal transduction in the guanylate cyclase pathway (24). This pattern of vascular dysfunction in subjects with type 2 diabetes is also consistent with a recent report in which the macrovascular (brachial artery) responses to flow-mediated endothelium-dependent dilation and sublingual nitroglycerin were both impaired in adults at risk for atherosclerosis (25). Although the long-term consequence of impaired microvascular reactivity is still not clear at present, it is now an accepted idea that the integrity of endothelial function is vital in maintaining vascular homeostasis (26). Therefore, our data would again suggest that the normoalbuminuric subjects with type 2 diabetes were already at risk for vasculopathy.

In summary, multiple markers of endothelial dysfunction were present in normoalbuminuric subjects with type 2 diabetes. Therefore, the pathogenic process of vasculopathy in type 2 diabetes occurs early and may be operative before the development of microalbuminuria.

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