Intensive treatment of risk factors in patients with type-2 diabetes mellitus is associated with improvement of endothelial function coupled with a reduction in the levels of plasma asymmetric dimethylarginine and endogenous inhibitor of nitric oxide synthase

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Aims Vascular endothelium is a major organ involved in hyperglycaemia and is affected by plasma asymmetric dimethylarginine (ADMA). ADMA is an endogenous, competitive inhibitor of nitric oxide synthase and is induced by inflammatory cytokines of tumour necrosis factor (TNF-α) in vitro. We hypothesized that a tight glycaemic control may restore endothelial function in patients with type-2 diabetes mellitus (DM), in association with modulation of TNF-α and/or reduction of ADMA level.

Methods and results In 24 patients with type-2 DM, the flow-mediated, endothelium-dependent dilation (FMD: %) of brachial arteries during reactive hyperaemia was determined by a high-resolution ultrasound method. Blood samples for glucose, cholesterol, TNF-α, and ADMA analyses were also collected from these patients after fasting. No significant glycaemic or FMD changes were observed in 10 patients receiving the conventional therapy. In 14 patients who were hospitalized and intensively treated, there was a significant decrease in glucose level after the treatment [from 190 ± 55 to 117 ± 21 (mean ± SD) mg/dL, P < 0.01]. After the intensive control of glucose level, FMD increased significantly (from 2.5 ± 0.9 to 7.2 ± 3.0%), accompanied by a significant (P < 0.01) decrease in TNF-α (from 29 ± 16 to 11 ± 9 pg/dL) and ADMA (from 4.8 ± 1.5 to 3.5 ± 1.1 μM/L) levels. The changes in FMD after treatment correlated inversely with those in TNF-α (R = −0.711, P < 0.01) and ADMA (R = −0.717, P < 0.01) levels.

Conclusion The intensive correction of hyperglycaemia is associated with the improvement of endothelial function, which is coupled with the decrease in the levels of reduction of plasma TNF-α and ADMA in patients with type-2 DM. A strict glycaemic control may exert anti-cytokine and anti-atherogenic effects and may therefore be pathophysiologically important.

Introduction
Cardiovascular disease is the major cause of morbidity and mortality in patients with type-2 diabetes mellitus (DM), in whom hyperglycaemia is one of the main metabolic abnormalities. Blood glucose control occupies the centre stage in DM management. A recent controlled trial, i.e. the United Kingdom Prospective Diabetes Study (UKPDS), suggested that an intensive glucose-lowering treatment decreases the occurrence of macrovascular complications. However, the exact roles of hyperglycaemia and glycaemic control in cardiovascular complications remain to be determined in patients with type-2 DM.

Previous studies demonstrated that acute hyperglycaemia impairs endothelium-dependent vasodilation in healthy subjects and further depresses it in patients with type-2 DM. These findings indicate a possible link between glucose level and endothelial function in humans. Endothelial dysfunction is an important phenomenon in the pathogenesis of atherosclerosis and is related to the derangements of nitric oxide synthase in the vessel wall. Asymmetric dimethylarginine (ADMA) is an endogenous, competitive inhibitor of NO synthase. Its concentration is increased by tumour necrosis...
factor-α (TNF-α),10 which is implicated as an important factor in the pathogenesis of type-2 DM.11
Thus, the present study was designed to investigate whether an intensive therapy of hyperglycaemia may improve endothelial function in association with the modula-
tion of the cytokines and/or decrease in plasma ADMA level in patients with type-2 DM.

Methods

Study patients

The study protocol was approved by the Institutional Review board, and all these patients gave their written informed consent to participate in the study. Type-2 DM was defined accord-
ing to the criteria of the Diagnosis and Classification of Diabetes Mellitus.12 Between May 1999 and June 2000, type-2 DM patients with poor glycaemic control [fasting blood glucose >200 mg/dL and/or haemoglobin A-1C (Hb A-1C) >9%] were recruited for intensive treatment of hyperglycaemia during hospitalization. Twenty-four patients were initially assessed for inclusion in the study. Among them, 14 patients [nine men and five women, mean age 61 ± 12 (SD) years] gave their consent and were admitted to the Hospital of the National Cardiovascular Center (intensive treatment group). The remaining 10 patients [seven men and three women, mean age 63 ± 15 (SD) years], who refused to be hospitalized and were obliged to keep conventional (non-intensive) diabetes treatment, served as the control group in the present study. All the patients underwent history screening, physical examination, and laboratory analysis, including a complete blood count: the levels of plasma electrolyte, glucose, insulin, Hb A-1C, blood urea nitrogen, creatinine, transaminases and urinary protein levels, and lipid profile. Moreover, the patients were assessed for the presence of diabetic complication, i.e. retinopathy, neuro-
pathy, nephropathy, a history of myocardial infarction, and the presence of angina pectoris and arteriosclerosis obliterans. Patients with nephrotic-range proteinuria, thyroid disease, appar-
tent infections, or haematologic, hepatic, or renal disease were excluded from the study. Before admission, five patients had been receiving angiotensin-converting enzyme inhibitors for hyper-
tension and five patients receiving statin for hyperlipidaemias for over 6 months. These medications were not changed throughout the study period. In addition, no new drugs other than insulin or oral hypoglycaemic agents were administered to any of these patients.

Study design

On admission, following an overnight fasting, a non-invasive assess-
ment of brachial arterial vasoreactivity in response to reactive hyperaemia or nitroglycerin was performed with blood sampling for the determination of the levels of glucose, insulin, Hb A-1C, total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, TNF-α, and ADMA in the plasma. We also measured plasma hepato-
cyte growth factor (HGF) level. The HGF may protect against endothelial dysfunction, and its production is suppressed by high glucose levels.13 Body mass index (BMI) was calculated using the formula BMI = weight (kg)/height² (m²). All measurements were repeated after ~1 month of intensive treatment for hyperglycaemia.

The intensive therapy was aimed at maintaining normal fasting glucose (80–115 mg/dL) and pre-prandial blood glucose (<130 mg/dL) levels. Throughout the study, the patients followed a 1200–1300 Kcal diet regimen of 60–65 g of protein, 30–35 g of fat, and 160–170 g of carbohydrates. The level of dietary choles-
terol was 350 g/day. The dose of oral anti-diabetic drugs was adjusted accordingly and/or insulin therapy was administered to improve glycaemic control. The patients were examined once or twice a week over a 4–5-week period of blood glucose monitoring. None of the patients experienced a hypoglycaemic reaction during the study.

Brachial artery ultrasound

Flow-mediated, endothelium-dependent vasodilation (FMD) following reactive hyperaemia and endothelium-independent nitroglycerin-induced vasodilation of the brachial artery were assessed using a high-resolution ultrasound machine (System Five, General Electronics) equipped with a 7.5 MHz linear array transducer.13 After a 10 min rest in a temperature-controlled room (22–23°C), the diameter of the right brachial artery and baseline forearm flow velocity were measured. Increased forearm blood flow was induced by the inflation of a blood pressure cuff placed around the forearm to 200 mmHg or to a pressure of 50 mmHg greater than the systolic blood pressure. This was followed by deflation (RD2 Cuff Deflator, Hokanson Inc., Bellevue, WA, USA) after 5 min. Repeated blood flow scans were obtained to measure the diameter of the brachial artery. After 15 min of vessel recup-
eration, a repeated measurement of the diameter of the resting brachial artery and repeated blood flow scans were obtained. Sublingual nitroglycerin (0.4 mg) was administered, and then final scans were obtained after 3 min. Throughout the study, a single lead electrocardiogram was obtained, and blood pressure was measured in the left arm every 2 min by an automated blood pressure recorder.

Ultrasound images were recorded on an S-VHS videocassette recorder. Depth and gain settings were used to optimize the images of the lumen-arterial wall interface. Vessel diameter was measured in triplicate at end diastole, from the anterior to the posterior interface between the media and the adventitia. Flow-
meditated vasodilation was calculated as the ratio of brachial artery diameter after reactive hyperaemia to baseline diameter and expressed as a per cent increase. Nitroglycerin-mediated vaso-
dilation was calculated in an analogous manner. Volumetric flow rate was calculated by multiplying the time velocity integral of the angle (~70°)-corrected Doppler flow signal by the heart rate and the vessel cross-sectional area. Changes in blood flow were expressed as the percentages of the resting flow measurements. All measurements were performed with the observers blind to patient information and study date. Using this methodology and analysis, the intra- and inter-observer variabilities in brachial artery diameter were 0.03 ± 0.02 (mean ± SD) and 0.06 ± 0.02 mm, respectively, and the variability in FMD performed on two different days was 1.4 ± 0.5%.

Laboratory measurements

Fasting plasma glucose level was measured by the glucose oxidase method and Hb A-1C level was measured by automated high-
performance liquid chromatography. Insulin level was measured by the conventional radioimmunoassay. To assess insulin resistance, we used the following homeostasis model assessment (HOMA) parameters: HOMA-R = [fasting blood glucose (mg/dL) × fasting insulin (µU/mL)]/405.14

Total cholesterol, triglyceride, and HDL cholesterol levels were determined as described previously.15 LDL cholesterol level was calculated using the Friedewald equation.16

TNF-α and HGF levels were determined by enzyme-linked immunoassorbent assay (Otsuka Pharmaceutical Co., Tokushima, Japan). The detection limits of these methods are 2 pg/mL for TNF-α and 0.1 ng/mL for HGF. The intra- and inter-assay coefficients of variation were both ~7% for the enzyme-linked immunoassorbent assay.

Plasma ADMA concentration was measured using high-
performance liquid chromatography with pre-column derivitiza-
tion, as previously described.17 In brief, equilibrated CBA columns
Results

The baseline characteristics of 10 control patients who received standard therapy and 14 intensively treated patients are summarized in Table 1. All 24 patients completed 3–4-week follow-up measurements.

The control patients were treated by diet alone (three patients) or diet plus oral hypoglycaemic agents (an increased dose of sulphonylurea, six patients and addition of metformin to sulphonylurea, one patient). Table 2 shows no significant improvements in clinical and biochemical parameters during the observation period of 28 ± 5 days of standard therapy. Neither the fasting blood glucose (from 181 ± 42 to 186 ± 38 mg/dL) nor the response of FMD to reactive hyperaemia (from 3.0 ± 1.3 to 2.6 ± 1.0%) changed.

Biochemical and clinical changes after intensive treatment of hyperglycaemia

In the intensive therapy group, the patients were all treated by diet alone (three patients), diet plus oral hypoglycaemic agents (sulphonylurea newly given, one patient; an increased dose of sulphonylurea, one patient; addition of metformin to sulphonylurea, two patients; and addition of α-glucosidase inhibitor to sulphonylurea, one patient), or diet plus insulin (switched from oral hypoglycaemic agents, six patients). The duration of intensive treatment of hyperglycaemia was 34 ± 13 days. Clinical and biochemical parameters at baseline (before treatment) were similar between the standard therapy group and the intensive therapy group (Tables 2 and 3). After the intensive...
treatment, the fasting glucose level significantly decreased from 190 ± 55 to 117 ± 21 mg/dL (P < 0.001), as shown in Figure 1. Significant decreases in Hb A-1C, total cholesterol, and LDL cholesterol levels and BMI were observed, whereas no changes in HOMA-R index; insulin, triglyceride, or HDL cholesterol levels; and systolic and diastolic blood pressures were observed (Table 3). Two of three patients with coronary artery disease were taking statins at the time of the study.

The levels of plasma TNF-α (from 29 ± 16 to 11 ± 9 pg/dL, P < 0.001) and ADMA (from 4.8 ± 1.5 to 3.5 ± 1.1 μM/L, P < 0.001) significantly decreased after the intensive control of glucose level (Figure 1). However, HGF level did not significantly change throughout the study (from 0.19 ± 0.05 to 0.20 ± 0.08 ng/mL).

### Brachial artery reactivity after intensive treatment of hyperglycaemia

Before treatment under hyperglycaemic condition, the baseline brachial arterial diameter was 4.5 ± 0.3 mm, and FMD in response to reactive hyperaemia was 2.4 ± 0.9%. After the intensive control of glucose level, FMD significantly increased to 7.2 ± 3.1% (Figure 1), whereas the baseline diameter (4.5 ± 0.2 mm) did not change. There was a similar increase in blood flow during reactive hyperaemia.
hyperaemia (293 ± 16 vs. 296 ± 20%) and a similar baseline heart rate (67 ± 7 vs. 65 ± 8 bpm) before and after the treatment.

Nitroglycerin-mediated vasodilation was 9.8 ± 1.0% before treatment; however, in contrast to FMD, it did not change after treatment (10.0 ± 1.6%).

Correlation with FMD improvement

As shown in Figure 2, the improvement of FMD after treatment correlated inversely with the changes in fasting glucose (R = −0.730, P < 0.01), TNF-α (R = −0.711, P < 0.01), and ADMA (R = −0.717, P < 0.01) levels. However, the improvement of FMD did not correlate significantly with the changes in Hb A-1C level (R = 0.408, P = 0.148), total cholesterol level (R = 0.325, P = 0.256), or BMI (R = 0.270, P = 0.351).

Six-to-12-month follow-up

A follow-up study was performed 6–12 months after the discharge. In eight of 14 patients with an Hb A-1C level of <8.0% at this follow-up period, fasting blood glucose level and FMD remained at 127 ± 26 mg/dL and 8.4 ± 1.0%, respectively. In contrast, in the remaining six patients with an Hb A-1C level of ≥8.0%, fasting blood glucose level and FMD worsened to be 178 ± 30 mg/dL and 3.1 ± 1.1%, respectively. There were inverse correlations of FMD with fasting blood glucose (R = −0.577) and Hb A-1C levels (R = −0.860).

Discussion

The major finding in the present study is that the intensive treatment of hyperglycaemia is associated with the improvement of endothelial function, coupled with the decrease in plasma TNF-α and ADMA (an endogenous inhibitor of NO synthase) levels in patients with type-2 DM.

Previous studies revealed that an acute increase in blood glucose level impairs endothelium-dependent vasodilation in healthy subjects and further inhibits it in patients with type-2 DM. DM is a state of chronic hyperglycaemia, and glycaemic control is one of the major goals of diabetes management. As shown in Figure 1, endothelial dysfunction improves after a 5-week intervention targeting hyperglycaemia in type-2 diabetes patients, accompanied by a relatively small but significant decrease in Hb A-1C level. In contrast, either hyperglycaemia or endothelial function did not change in control outpatients who received routine treatment. These findings suggest that hyperglycaemia may be a fundamental abnormality underlying the mechanism that causes endothelial dysfunction in DM. However, we must acknowledge a potential limitation that an appropriate control group should have included patients who were admitted to the hospital, but did not receive intensive treatment. In addition, the number of statistical tests performed and relatively small sample size of the study population may potentially infiltrate type-I error.

In patients with type-2 DM, TNF-α levels were elevated in both blood and tissue. Taken together with results from knockout mice deficient in TNF-α or its receptors, it is suggested that TNF-α is a factor contributing to the pathogenesis of type-2 DM. Hyperglycaemia is an important stimulus for TNF-α synthesis in human peripheral monocytes in vitro. A previous in vivo study demonstrated that the administration of TNF-α impairs endothelial-dependent vasodilation in rats. In the present study, as shown in Figure 1, plasma TNF-α level decreased after the intensive treatment of hyperglycaemia. This finding indicates the therapeutic potential of a strict glycaemic control against inflammatory cytokines that play a prominent role in atherogenesis.
TNF-α and hyperglycaemia could impair dimethylarginine dimethylamino-hydrolase and cause the accumulation of ADMA, an endogenous, competitive inhibitor of NO synthase, contributing to the derangements of NO pathways in the vessel.10,24 The intra-arterial infusions of ADMA significantly impair endothelium-dependent flow responses in the human forearm.25 In the present study, we found that the ADMA level increased in patients with type-2 DM (Figure 1), and its decrease after the strict glycaemic control correlated significantly with the improvement of FMD (Figure 2). Not only ADMA, but also TNF-α itself downregulates NO synthase by decreasing mRNA’s half-life.26 Moreover, both inflammatory cytokines and high glucose levels enhance the production of oxygen-derived free radicals,27,28 which rapidly inactivate NO.29 In patients with type-2 DM, the extent of urinary excretion of the isoprostanes (8-iso-prostaglandin F₂α) significantly decreased ~4 weeks after an intensive therapy for hyperglycaemia, an intervention similar to that used in the present study.30 Taking together a recent report that lowering serum TNF-α level alone (without glycaemic control) does not improve endothelial function,31 these findings suggest that the hyperglycaemia-induced oxidative stress could be a key factor in the pathophysiology of diabetes.

HGF is characterized to be one of the most potent mitogens among the growth factors for vascular endothelial cells and contributes to vascular protection or repair.13 Because its production is suppressed by glucose in a dose-dependent manner in vitro,14 we hypothesized that endothelial dysfunction might be associated with the decreased production of HGF in diabetic patients. However, this was not the case. The level of HGF did not change throughout this study. Moreover, as shown in Table 3, insulin sensitivity, as assessed using HOMA-R index,14 did not change significantly. Insulin resistance contributes, in part, to the pathogenesis of type-2 DM and may be potentially linked with endothelial dysfunction and ADMA.32 To address this important issue, we need to further assess insulin sensitivity with a more specific method such as steady-state plasma glucose measurement.

Impaired endothelium is a key factor for diabetic macroangiopathy.7 Thus, restoring endothelial function has important clinical implications for reducing the risk of cardiovascular diseases in diabetic patients. The present results, although obtained in a short period, suggest that a long-term maintenance of strict glycaemic control is important. If hyperglycaemia continues, then the expression level of NO synthase and the generation of NO may be chronically reduced, leading to a persistent dysfunction of the vascular endothelium and the consequent atherogenesis. In the UKPDS conducted for more than 15 years,4 the difference in Hb A-1C level between the conventionally and intensively treated groups was significant throughout the study. However, Hb A-1C level progressively increased in both groups. The median Hb A-1C level was 6.6% in the first 5 years, but increased to 8.1% in the last 5 years, even in the intensively treated group. A difficulty in maintaining a good glycaemic control may explain, in part, the borderline decrease in the extent of myocardial infarction (P = 0.05) induced by the intensive treatment. Taking the multifactorial aetiology of macrovascular disease into account, the results of the UKPDS also suggest that the optimum treatment of patients with type-2 DM would include the control of blood pressure and correction of lipid abnormalities in addition to the control of glucose level. For the assessment of the effectiveness of therapeutic/dietary interventions and for the early detection of vascular dysfunction, plasma ADMA may be useful as a potential biochemical marker.9,33 Metformin,34 angiotensin-converting enzyme inhibitors/angiotensin II receptor blocker,35 and statins36 could decrease ADMA level. Although these drugs were not newly given in the present patients, it is possible that an increased utilization of and compliance with medications and an improved diet during hospitalization may contribute, at least in part, to endothelial function improvement. Insulin-sensitizing rosiglitazone also decreases ADMA level.37 A recent study has suggested that obese and insulin resistance are not strongly associated with the development of type-2 DM in Japanese patients with a BMI of ~23 kg/m² (from the Japan Diabetes Complications Study), unlike in European patients with a BMI of ~29 kg/m² (from the UKPDS).38

In conclusion, in patients with type-2 DM, the intensive treatment of hyperglycaemia is associated with the improvement of endothelial dysfunction, coupled with decreases in TNF-α and ADMA levels. A strict glycaemic control may exert anti-cytokine and anti-atherogenic effects and may therefore be pathophysiologically important.

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Conflict of interest: none declared.

References


