Tissue Factor Pathway Inhibitor Activity in Patients With IDDM

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Until now, several endothelium-dependent hemostatic parameters have been proposed as markers of vascular endothelial dysfunction in diabetes. We studied tissue factor pathway inhibitor (TFPI) activity in insulin-dependent diabetes mellitus (IDDM) patients without macro- or microvascular complications, before and after intravenous administration of heparin, in comparison with age-matched control subjects. We also examined the effect of acute hyperglycemia on TFPI activity in healthy men. A clotting and a chromogenic assay were used for determining TFPI activity. In the clotting assay, the COOH-terminus of TFPI is essential, but in the chromogenic assay, it is of minor importance. When the chromogenic assay was used, TFPI activity before heparin injection was significantly higher in the IDDM patients (92 ± 24 vs. 112 ± 23%, $P < 0.01$). The postheparin increase in TFPI activity, measured with both assays, was significantly higher in the diabetic subjects (area under the curve: clotting assay 64 ± 14 vs. 81 ± 24, $P < 0.05$; chromogenic assay 82 ± 26 vs. 121 ± 35, $P < 0.0001$). A positive correlation between TFPI activity and glycated hemoglobin was demonstrated. Acute hyperglycemia did not alter TFPI activity. It can be concluded that TFPI activity, especially after stimulation with heparin, is affected by chronic hyperglycemia in diabetic subjects without vascular complications. Alterations in TFPI activity may therefore reflect early endothelial dysfunction. *Diabetes* 44:80–84, 1995

Diabetes is associated with premature atherosclerosis. It is known that endothelial cells play an important role in the repair process after vascular injury, thereby maintaining the functional integrity of the vascular wall. Endothelial damage has been considered to be one of the initiating events in the pathogenesis of atherosclerosis (1).

The vascular endothelium can be considered a paracrine organ that regulates hemostasis (coagulation and fibrinolysis). It produces procoagulant substances (e.g., von Willebrand factor [vWF] and tissue factor) and components of the fibrinolytic system that promote or inhibit fibrinolysis (tissue plasminogen activator [t-PA] and plasminogen activator inhibitor-1 [PAI-1], respectively) (2,3). Endothelium also expresses factors associated with anticoagulant properties, such as heparan sulfate and thrombomodulin (2,3). In 1987, a new Kunitz-type coagulation inhibitor, tissue factor pathway inhibitor (TFPI), was identified as a single protein (4). Coagulation activity, initiated by the tissue factor pathway, is activated by the complex of tissue factor and factor VIIa. This complex activates not only factor X but also factor IX, via the Josso loop (5,6). TFPI directly inhibits factor Xa and, in complex with factor Xa, the tissue factor/factor VIIa catalytic complex (7). The major site of production of TFPI is the vascular endothelium (8). Of TFPI, 50–90% is found on the endothelium, 10–50% in plasma, and the remainder in platelets (9). Most TFPI in the plasma is associated with lipoproteins (10,11). Only ~5% of the TFPI in the plasma circulates as a free, uncomplexed protein (9). TFPI activity is altered in several pathological conditions (12–14). Its release from the endothelium can be stimulated by heparin (15).

Increased levels of vWF, thrombomodulin, t-PA, and PAI-1 are found in diabetic patients with microvascular complications (16–18). It is therefore suggested that these alterations in endothelium-dependent hemostatic parameters reflect vascular endothelial damage. Little is known about TFPI activity in diabetic subjects, however. Because TFPI may also be a marker of endothelial dysfunction, we studied TFPI activity in insulin-dependent diabetes mellitus (IDDM) patients without vascular complications, before and after intravenous administration of heparin. The effect of acute hyperglycemia on TFPI activity was investigated in healthy men. We also examined the relationship between the degree of metabolic regulation and TFPI activity.

RESEARCH DESIGN AND METHODS

Twenty-five IDDM patients (12 men, 13 women; mean age 33 [range 19–49 years]) and 21 age-matched healthy control subjects (10 men, 11 women; mean age 33 [range 22–43 years]) were studied. Only IDDM subjects without neuropathy, retinopathy, nephropathy, or macrovascular complications were included. Thus, all patients were normoalbuminuric and normotensive. Patients were also divided according to degree of metabolic control. The characteristics of the subjects are depicted in Table 1. Each study started at 8:00 A.M. while the subject was still fasting. With the subject in supine position, blood samples were taken via an indwelling catheter in a forearm vein at 15 and 0 min before and 3, 5, 10, and 30 min after an intravenous bolus injection of 5,000 IU of unfractionated heparin (Leo, Denmark) in the contralateral forearm. For determining TFPI activity, vWF, thrombomodulin, t-PA, and PAI-1, blood samples were collected in tubes containing 3.25% sodium citrate (dilution 1:10). Blood samples were also drawn to determine glycated hemoglobin and lipid profiles at baseline. Glycated hemoglobin (HbA_{1c} fraction) was measured immediately by high-performance liquid chromatography (Diamat, Bio-Rad, U.S.), the remainder of the blood samples were centrifuged, and plasma was stored at −70°C until further processing.

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Received for publication 21 March 1994 and accepted in revised form 5 October 1994.

vWF, von Willebrand factor; t-PA, tissue plasminogen activator; PAI, plasminogen activator inhibitor; TFPI, tissue factor pathway inhibitor; IDDM, insulin-dependent diabetes mellitus; CV, coefficient of variation; AUC, area under the curve; HDL, high-density lipoprotein; apo, apolipoprotein; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); ANOVA, analysis of variance; PF4, platelet factor 4; AGE, advanced glycation end product.

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TABLE 1
Demographic and laboratory parameters of IDDM patients and control subjects at baseline

	Control subjects	IDDM patients		
		Overall	HbA_{1c} < 7.4%	HbA_{1c} ≥ 8.4%
	I	II	III	IV
n	21	25	12	13
Sex ratio (M/F)	10/11	12/13	6/6	6/7
Age (years)	33 ± 6	33 ± 8	35 ± 7	32 ± 8
Duration diabetes (years)	—	9 (1–31)	9 (1–31)	9 (1–21)
Dosage insulin (IU/day)	—	48 ± 17	43 ± 15	53 ± 16
Body mass index (kg/m^2)	23.7 ± 2.1	23.3 ± 2.5	22.7 ± 2.8	23.8 ± 2.0
Glycated hemoglobin (%)	4.9 ± 0.4*	8.1 ± 1.6	6.7 ± 0.5†	9.5 ± 0.9
Total cholesterol (mmol/l)	5.0 ± 0.7	5.3 ± 1.4	4.9 ± 0.6	5.8 ± 1.7
Triglycerides (mmol/l)	0.87 (0.24–2.08)	1.12 (0.5–3.56)	0.98 (0.5–3.56)	1.12 (0.76–1.66)
HDL cholesterol (mmol/l)	1.34 ± 0.33	1.43 ± 0.42	1.41 ± 0.45	1.46 ± 0.40
LDL cholesterol (mmol/l)	3.21 ± 0.63	3.32 ± 1.46	2.81 ± 0.67	3.78 ± 1.78
Apo A_1 (g/l)	1.51 ± 0.18	1.60 ± 0.22	1.60 ± 0.25	1.61 ± 0.21
Apo B (g/l)	0.93 ± 0.22	1.05 ± 0.33	0.94 ± 0.17	1.14 ± 0.39
Lp(a) (U/l)	139 (17–1478)	126 (12–1714)	126 (21–848)	143 (12–1714)
Serum insulin (pmol/l)	41.4 (19.8–69)	49.2 (18–189)	45 (22.2–189)	49.2 (18–149.4)

Data are means ± SD or medians (ranges). *$P < 0.05$ vs. II, III, and IV; †$P < 0.05$ vs. IV.

A second study regarding the effect of an acute glucose load on TFPI activity was performed in six healthy men (mean age 30 [range 24–36 years]; mean body mass index 23.1 ± 1.4 kg/m^2). After an overnight fast, with the subject in supine position, blood samples were taken before and 10 and 30 min after intravenous administration of 50% glucose (0.5 g/kg body wt with a maximum of 35 g) for 3 min in the contralateral forearm. Blood samples for glucose, insulin, and C-peptide determinations were also taken. Both protocols were approved by the ethical committee of the University Hospital. Written informed consent was obtained from all participants.

TFPI activity was measured using both a modified clotting assay based on the diluted tissue thromboplastin clotting time as reported previously (expressed in seconds; coefficient of variation [CV] 4.5%) (19) and the chromogenic substrate assay according to Sandset et al. (20) (expressed in percentage with respect to standardized TFPI activity, measured in a plasma pool obtained from 45 healthy donors; CV 7.4% at the 100% level). The tissue factor concentration, used in the clotting assay, was adjusted to a coagulation time of 123 s measured in normal plasma (19). With this tissue factor concentration, an optimal balance between the accuracy and sensitivity of the clotting assay was achieved. Two different assays were used because with the clotting method, mainly full-length TFPI, which has a very strong bioactivity, is measured, while the chromogenic method also measures COOH-terminal fragmented TFPI, which has only a weak anticoagulant activity. The area under the curve (AUC) for TFPI activity was also calculated. Plasma levels of vWF were determined by enzyme-linked immunosorbent assay using rabbit anti-human vWF (Dako A/S, Denmark; CV 7%; normal range 60–180%). Because of the well-known relation between vWF and blood group O (21), the blood of the study population was typed. Thrombomodulin determination was performed with an enzyme immunoassay (Diagnostica Stago, France; CV 8–10%; normal range 50–120 ng/ml, mean 76 ± 22 ng/ml). An enzyme immunoassay was also used for the measurement of t-PA antigen (Innogenetics, Belgium; CV 7%; normal range 1.3–10.4 ng/ml, mean 4.1 ± 2.4 ng/ml). PAI-1 activity was photometrically measured (Kabi Diagnostica, Sweden; intra-assay CV 0.4–2.4%; normal range 1–20 AU/ml, median 8 AU/ml). Total and high-density lipoprotein (HDL) cholesterol and triglycerides were determined with enzymatic colorimetric tests (Unimate 5 and 7, Roche, Switzerland), and apolipoprotein (apo) A_1 and apo B were measured with immunoturbidimetry (Uni-kit, Roche). Low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula. Lipoprotein(a) [Lp(a)] was determined by a solid-phase, two-site immunoradiometric assay using two monoclonal antibodies directed toward different epitopes of apo(a) (Pharmacia, Sweden). At an Lp(a) concentration of 200 U/l, the within-assay CV was 4%. An enzymatic hexokinase method (Unimate 5, Roche) was used for measuring serum glucose concentrations. Free serum insulin was measured with a double antibody radioimmunoassay (Pharmacia; within-assay CV 3.4–6.1% in the range of 18–300 pmol/l) after polyethylene glycol pretreatment. C-peptide concentration in serum was also determined by radioimmunoassay (Byk-Sangtec, Germany; within-assay CV 1.9–5.0% in the range of 0.10–1.75 nmol/l).

Statistical analysis. All data are expressed as means ± SD or as median and range when not normally distributed. Differences between two group means were tested with Student's *t* test. When data were not normally distributed, the Mann-Whitney-Wilcoxon rank-sum test was applied. To analyze postheparin TFPI changes, multiple analysis of variance (ANOVA) for repeated measurements was performed. For comparing more than two groups, one-way ANOVA with Student-Newman-Keuls correction for multiple comparisons was used. The Kruskal-Wallis ANOVA was applied if data were not normally distributed. $P \leq 0.05$ was considered statistically significant.

RESULTS

The IDDM patients and control subjects were well-matched in terms of demographic parameters (Table 1). There were also no significant differences between the well-controlled and poorly controlled diabetic subjects with respect to duration of diabetes and daily insulin dosage. Lipid profile, Lp(a), apo A_1, apo B, and insulin concentrations were similar in the diabetic and control groups, and there was no significant correlation between these apo, lipoprotein, or serum insulin levels and the endothelium-dependent indexes. Regarding the endothelium-dependent coagulant and fibrinolytic proteins at baseline, plasma levels of thrombomodulin and PAI-1 activity were not different between the study groups (Table 2). In contrast, t-PA antigen was significantly lower ($P < 0.01$) in the diabetic subjects than in the control subjects, independent of the degree of metabolic control, but the plasma level of vWF was higher ($P < 0.01$). However, levels of t-PA antigen and vWF in the diabetic patients were still within normal ranges. A significant correlation between t-PA and TFPI activity was found when measured with the clotting assay ($r = 0.56$, $P < 0.01$). There was no difference in blood groups between the control and diabetic subjects. TFPI activity before administration of heparin was similar in the various groups when it was measured with the clotting assay. In contrast, when measured chromogenically, TFPI activity was significantly higher ($P < 0.01$) in the diabetic group at baseline. In poorly controlled patients, TFPI activity was higher ($P < 0.05$) than in well-controlled subjects (Fig. 1). The postheparin TFPI activity in the diabetic subjects was

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TABLE 2
Hemostatic parameters of IDDM patients and healthy subjects at baseline

	Control subjects	IDDM patients		
		Overall	HbA_{1c} < 7.4%	HbA_{1c} ≥ 8.4%
	I	II	III	IV
VWF (%)	84.7 ± 24.3*†	103.5 ± 21.8	109.0 ± 22.3	98.5 ± 21.0
Thrombomodulin (ng/ml)	80.1 ± 22.7	80.6 ± 22.3	85.2 ± 20.5	76.5 ± 23.9
t-PA (ng/ml)	8.77 ± 2.61*‡	6.65 ± 2.23	6.82 ± 2.42	6.49 ± 2.11
PAI-1 (AU/ml)	6.9 (1.0–25.9)	4.3 (1.0–16.0)	5.0 (1.0–15.9)	2.9 (1.0–16.0)
TFPI activity by clotting assay (s)	116 ± 10	116 ± 11	115 ± 10	117 ± 11
TFPI activity by chromogenic assay (%)	92 ± 24*	112 ± 23	101 ± 12	122 ± 25§

Data are means ± SD or medians (ranges). *$P < 0.01$ vs. II; †$P < 0.05$ vs. III; ‡$P < 0.05$ vs. III and IV; §$P < 0.05$ vs. I and III.

higher than in the control subjects. The difference in increase of TFPI activity, measured chromogenically, was significant at all points after intravenous administration of heparin ($P < 0.0001$). When diabetes control was poor, the postheparin increase of TFPI activity was significantly higher than when it was good (at 5 min, 264 ± 95% vs. 352 ± 73%, $P < 0.05$; Fig. 1). The maximum peak in TFPI activity in the IDDM group (421 ± 106%) and control subjects (293 ± 71%) was seen 5 min after heparin administration. A significant correlation was found between HbA_{1c} and the AUC of postheparin TFPI activity ($r = 0.68$, $P < 0.001$; Fig. 2). When applying the clotting assay, comparable results were seen. The increase in TFPI activity was significantly higher in the diabetic subjects at 5 to 30 min postheparin ($P < 0.05$). A correlation also existed between the AUC of postheparin TFPI activity and HbA_{1c} ($r = 0.45$, $P < 0.001$).

In the second study, the mean fasting glucose concentration was 4.2 ± 0.2 mmol/l, which increased to a maximum of 16.1 ± 1.4 mmol/l 1 min after intravenous administration of glucose. After 10 and 30 min, the glucose levels were 11.9 ± 0.8 and 7.2 ± 1.9 mmol/l, respectively (Table 3). TFPI activity, measured with both assays, did not change after induction of acute hyperglycemia.

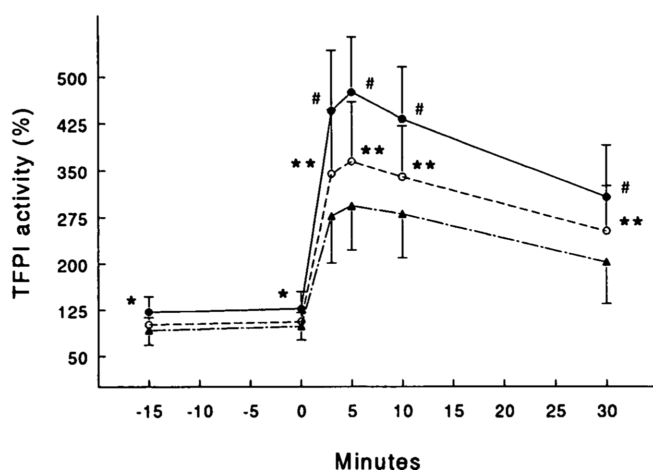

FIG. 1. The increment of TFPI activity measured chromogenically after intravenous bolus injection of 5,000 IU of heparin in well- (○; $n = 12$) and poorly (●; $n = 13$) regulated IDDM patients and healthy control subjects (▲; $n = 21$). Before administration of heparin, TFPI activity is significantly higher in the poorly regulated patients (*$P < 0.05$ vs. well-regulated group, $P < 0.01$ vs. control subjects). The difference in increase in postheparin activity is already significant 3 min after stimulation (#$P < 0.05$ vs. well-regulated group, $P < 0.0001$ vs. control subjects; **$P < 0.05$ vs. control subjects).

DISCUSSION

TFPI is considered an important protein in the regulation of hemostasis. In addition, its activity may reflect endothelial function, because TFPI is produced by and mainly found on vascular endothelium. It is possibly bound to the endothelial cell surface by glycosaminoglycans (9). The COOH-terminus of TFPI, which is important for binding glycosaminoglycans like heparin, can be truncated by various enzymes, resulting in considerable loss of its anticoagulant activity (22,23). In the present study, two different assays for determining TFPI activity were used. The modified clotting assay measures the anticoagulant full-length TFPI, but the chromogenic assay also measures COOH-terminal truncated TFPI. In comparison with healthy control subjects, a significantly higher increase in TFPI activity after stimulation with heparin was observed in our IDDM subjects. This was true for both assays. When applying the clotting assay, the increase in TFPI activity appeared to be independent of the activity at baseline.

It is well known that in diabetic subjects several hemostatic parameters are disturbed. These include coagulant and fibrinolytic components that, like TFPI, are dependent on the vascular endothelial cell function (24,25). Our results indicate that TFPI activity after stimulation with heparin is influenced by a chronic hyperglycemic condition. Not only was a higher increase in TFPI activity seen in the diabetic

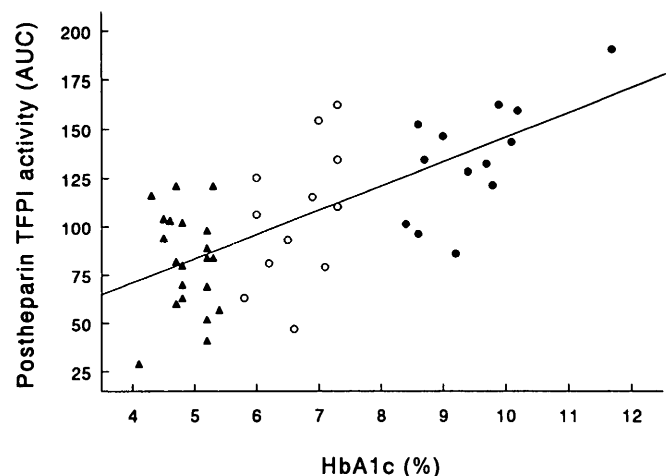

FIG. 2. A significant correlation ($r = 0.68$, $P < 0.001$) is found between the degree of metabolic regulation and the AUC of chromogenically measured postheparin TFPI activity. When the clotting assay was used, a weaker correlation was found ($r = 0.45$, $P < 0.001$). ▲, healthy control subjects; ○, well-regulated IDDM patients; ●, poorly regulated IDDM patients.

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TABLE 3
Effect of an acute glucose load on TFPI activity in six healthy males

	0 min	10 min	30 min
Glucose (mmol/l)	4.2 ± 0.3*	11.9 ± 0.8†	7.2 ± 1.9
Insulin (pmol/l)	29.4 (9.6–65.4)‡	132 (61.6–316.2)	82.8 (58.2–228)
C-peptide (nmol/l)	0.77 ± 0.29§‖	2.20 ± 0.77	2.04 ± 0.70
TFPI activity by clotting assay (s)	125 ± 6	128 ± 4	127 ± 7
TFPI activity by chromogenic assay (%)	115 ± 18	106 ± 18	106 ± 21

Data as means ± or medians (ranges). *$P < 0.0001$ vs. 10 min and 30 min; †$P < 0.0001$ vs. 30 min; ‡$P < 0.01$ vs. 10 min and 30 min; §$P < 0.001$ vs. 10 min; ‖$P < 0.005$ vs. 30 min.

patients, but a significant positive correlation between postheparin TFPI activity and HbA_{1c} was found. No relation between TFPI activity and serum insulin levels could be demonstrated, nor did acute hyperglycemia have any effect on basal TFPI activity in healthy subjects.

It is known that TFPI has an association with the lipoproteins LDL, HDL, and Lp(a) (10,11). In the present study, mean total and LDL cholesterol and the median of Lp(a) levels tended to be higher in poorly regulated diabetic patients than in the well-regulated diabetic and control groups, but this difference did not reach statistical significance. We did not find any correlation between TFPI activity at baseline or postheparin and plasma apo and lipoproteins.

An important feature of diabetes is the occurrence of nonenzymatic glycation reactions in which proteins, important in hemostasis, are involved (26). The difference in the measurements of TFPI activity between the clotting and chromogenic methods, especially at baseline, could be the result of a difference in sensitivity between the assays. However, it may also suggest the presence of fragmented TFPI in diabetic patients. This may be the result of nonenzymatic glycation. Additionally, TFPI is thought to be bound to the endothelial cell surface by glycosaminoglycans (9). Heparin is able to release proteins that are bound to glycosaminoglycans, such as platelet factor 4 (PF4), from the endothelium (27). In diabetic subjects, the release of PF4 after intravenous administration of heparin was significantly higher than in healthy control subjects (27). Heparin probably binds to lysine residues as in antithrombin III, resulting in a more rapid reaction with coagulation proteases (28). It has been demonstrated that this heparin-cofactor activity of antithrombin III is altered by nonenzymatic glycation (26). As already mentioned, the COOH-terminus of TFPI, which contains lysine residues, is essential for binding to heparin. We speculate that in diabetes, the COOH-terminus of TFPI is affected by the process of nonenzymatic glycation. This may lead to functional alterations of these molecules, resulting in an altered binding between glycosaminoglycans and TFPI, which may be responsible for the excess of release of TFPI in diabetic patients after injection of heparin. An additional explanation may be the nonenzymatic glycation of apo A_1 and apo B (29,30). The COOH-terminus of TFPI thereby appears to be involved in the interaction between TFPI and the lipoproteins (31). It has been shown that glycation diminishes the interaction between apo A_1 and the HDL particle (30). If one presumes that this also occurs between the lipoproteins and TFPI, one may expect a redistribution of TFPI in favor of the vascular endothelium. In that case, a higher response in TFPI activity after heparin injection may occur.

Nonenzymatic glycation eventually results in the formation and accumulation of advanced glycation end products (AGEs). AGEs are able to affect the endothelial cellular function by interacting with specific receptors, inducing an increase in tissue factor activity and a reduction in thrombomodulin activity (32). The altered TFPI activity in diabetes may also be related to functional alterations in the endothelium by AGEs.

Increased oxidative stress as seen in diabetes is accompanied by abnormalities in hemostatic parameters, especially in subjects with microalbuminuria (18,33). Collier et al. (18) observed elevated levels of vWF, t-PA, and PAI-1, suggesting that these hemostatic alterations were a result of damage to endothelial cells. Because our patients had no secondary complications, the increase in postheparin TFPI activity may be a reflection of early endothelial damage. There was a significant difference between our diabetic and control subjects with respect to vWF and t-PA. This contrasts with earlier reports, most of which suggested that vWF and t-PA levels are only changed in diabetic patients with microvascular complications (16,18). Because the levels of t-PA and vWF in our patients were still within normal ranges, the exact significance of these altered levels is not clear. The differences in levels of vWF could not be explained by a different distribution of blood group. There was no correlation between TFPI activity and vWF. However, a significant correlation between t-PA and TFPI activity existed when measured with the clotting assay. The explanation for this finding is unclear, especially because the t-PA levels were significantly lower in the IDDM group in comparison with the control group. Although metabolic control may influence the levels of t-PA and vWF (34,35), we found no relation between t-PA and vWF and HbA_{1c}.

In conclusion, postheparin TFPI activity in IDDM patients without secondary complications is significantly increased and correlates with the degree of metabolic derangement. The explanation for this observation is not yet known, but nonenzymatic glycation and AGE formation may play an important role. However, early damage of endothelial vascular cells as a direct result of hyperglycemia and oxidative stress cannot be ruled out.

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