Simultaneous Control of Hyperglycemia and Oxidative Stress Normalizes Endothelial Function in Type 1 Diabetes

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OBJECTIVE — Previous studies have shown that in type 1 diabetes endothelial dysfunction persists even when glycemia is normalized. Moreover, oxidative stress has recently been demonstrated to be the mediator of hyperglycemia-induced endothelial dysfunction.

RESEARCH DESIGN AND METHODS — Thirty-six type 1 diabetic patients and 12 control subjects were enrolled. The diabetic patients were divided into three groups. The first group was treated for 24 h with insulin, achieving a near-normalization of glycemia. After 12 h of this treatment, vitamin C was added for the remaining 12 h. The second group was treated for 24 h with vitamin C. After 12 h of this treatment, insulin was started, with achievement of near-normalization of glycemia for the remaining 12 h. The third group was treated for 24 h with both vitamin C and insulin, achieving near-normalization of glycemia.

RESULTS — Neither normalization of glycemia nor vitamin C treatment alone was able to normalize endothelial dysfunction or oxidative stress. However, a combination of insulin and vitamin C normalized endothelial dysfunction and decreased oxidative stress to normal levels.

CONCLUSIONS — This study suggests that long-lasting hyperglycemia in type 1 diabetic patients induces permanent alterations in endothelial cells, which may contribute to endothelial dysfunction by increased oxidative stress even when hyperglycemia is normalized.

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Type 1 diabetes is associated with increased incidence of macrovascular diseases (1).The accelerated macrovascular disease is due in part to the increased incidence of classic risk factors, such as hypertension and dyslipidemia (2). However, recent evidence suggests that hyperglycemia also plays a significant role (3).

The endothelium is a major organ involved in the development of cardiovascular disease, even in diabetes (4). All risk factors involved in the pathogenesis of cardiovascular disease, such as dyslipidemia and hypertension, can induce endothelial dysfunction, which has largely been shown to be predictive of a future cardiovascular event (4).

The presence of endothelial dysfunction has often been reported in diabetes (4). However, whereas several studies have shown that hyperglycemia induces endothelial dysfunction in both diabetic and nondiabetic subjects (5–7), a clear demonstration that control of hyperglycemia can restore/normalize endothelial function is still lacking. In particular, in type 1 diabetic patients endothelial dysfunction has been reported to be present even when normoglycemia was achieved (8,9). Furthermore, several studies indicated that hyperglycemia induces endothelial dysfunction through the generation of oxidative stress (for review, see ref. 10), which has been suggested to be the key player in the generation of diabetes complications, both micro- and macrovascular (11). Therefore, the aim of this study was to evaluate the distinct as well as the combined effect of controlling hyperglycemia and oxidative stress on endothelial function in type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

— Experiments were performed in 36 type 1 diabetic patients; 12 age- and sex-matched healthy volunteers served as a control group (Table 1). The diabetic subjects were divided into three subgroups, matched for sex, age, BMI, metabolic control, and duration of the disease (Table 1). None of the selected patients were hypertensive or microalbuminuric or were receiving drug treatment, excluding insulin. Lipid levels were also normal. The study was approved by the local ethics committee, and all subjects gave written informed consent.

All experiments were performed in a quiet, temperature-controlled room (24–25°C) after an overnight fast. 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. In the diabetic patients, a catheter was inserted into an antecubital vein for the infusion of one of the three treatment protocols:

A. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) and/or 5% glucose to keep blood glucose levels between 4 and 6 mmol/l for 24 h. The amount of insulin infused was based on the individual daily insulin dose.

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All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Abbreviations: AGE, advanced glycation end product; ELISA, enzyme-linked immunosorbent assay; FMD, flow-mediated vasodilatation; NO, nitric oxide; NOS, nitric oxide synthase; PARP-1, poly(ADP-ribose) polymerase-1; PKC, protein kinase C; TNM, tetranitromethane.

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

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Table 1—*Baseline characteristics of control subjects and type 1 diabetic patients enrolled in protocols A, B, and C (see* RESEARCH DESIGN AND METHODS*)*

	Control subjects	Protocol A	Protocol B	Protocol C
Sex (male/female)	6/6	7/5	5/7	7/5
Age (years)	27.4 ± 3.2	27.8 ± 3.2	25.8 ± 4.1	26.6 ± 3.5
Disease duration (years)		8.8 ± 6.2	9.2 ± 6.4	8.9 ± 7.1
BMI (kg/m^2)	23.8 ± 2.2	22.7 ± 2.1	23.5 ± 2.2	22.4 ± 2.3
Fasting glucose (mmol/l)	4.3 ± 0.3	8.2 ± 2.3*	8.1 ± 3.2*	7.8 ± 2.4*
A1C (%)		8.3 ± 0.3	8.2 ± 0.5	8.3 ± 0.5
Resting systolic blood pressure (mmHg)	119.1 ± 3.0	120.4 ± 2.5	125.3 ± 2.1	123.2 ± 1.5
Resting diastolic blood pressure (mmHg)	80.4 ± 2.1	81.2 ± 3.1	82.6 ± 2.1	82.4 ± 3.7
Total cholesterol (mmol/l)	4.8 ± 0.6	4.9 ± 0.8	4.9 ± 0.9	5.0 ± 0.6
Triglycerides (mmol/l)	1.1 ± 0.5	1.3 ± 0.4	1.3 ± 0.5	1.2 ± 0.4
HDL cholesterol (mmol/l)	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.4	1.4 ± 0.2
LDL cholesterol (mmol/l)	2.6 ± 0.5	2.5 ± 0.4	2.5 ± 0.4	2.6 ± 0.6
FMD (%)	10.8 ± 0.8	5.5 ± 0.5*	5.6 ± 0.7*	5.6 ± 0.8*
Nitrotyrosine (μmol/l)	0.35 ± 0.3	0.73 ± 0.2*	0.71 ± 0.4*	0.72 ± 0.3*

Data are means ± SEM. *$P < 0.001$ vs. control subjects.

Blood glucose levels were determined every 5 min with adjustment of the intravenous insulin infusion until steady-state glucose levels were between 4 and 6 mmol/l. At the steady state, venous glucose samples were drawn every 30 min. After 12 h, vitamin C infusion, at the rate of 3 mg/min (12), was also started and continued for the remaining 12 h.

B. Vitamin C, at the rate of 3 mg/min (12), for 24 h. After 12 h, insulin and/or a 5% glucose infusion was added with the aim of maintaining blood glucose levels between 4 and 6 mmol/l, as described above, for the remaining 12 h.

C. Insulin and/or 5% glucose to keep blood glucose levels between 4 and 6 mmol/l plus vitamin C infusion at the rate of 3 mg/min (12), for 24 h.

At baseline and after 12 and 24 h, glycemia, endothelial function (flow mediated dilatation [FMD]), and nitrotyrosine plasma levels were evaluated.

Biochemical measurements

Cholesterol and triglycerides were measured enzymatically (Roche Diagnostics, Basel, Switzerland). HDL cholesterol was estimated after precipitation of apolipoprotein B with phosphotungstate-magnesium (13). LDL cholesterol was calculated after lipoprotein separation (14). Plasma glucose was measured by the glucose oxidase method and A1C by high-performance liquid chromatography.

Nitrotyrosine

The nitrotyrosine plasma concentration was assayed by an enzyme-linked immunosorbent assay (ELISA), developed according to Ter Steege et al. (15) and recently validated by our laboratory (16). BSA, tetranitromethane (TNM), IgG secondary antibody, and tetramethylbenzidine were purchased from Sigma-Aldrich (St. Louis, MO). Maxisorp ELISA plates were from NUNC, Life Technologies (Grand Island, NY), and IgG monoclonal anti-nitrotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY).

Preparation of nitrated protein. Nitrated protein solution was prepared by incubating 1 mg/ml BSA in 50 mmol/l KH_2PO_4 at pH 7.4 for 30 min at 37°C with 1 mmol/l TNM, an efficient nitrating agent (17). After adjusting the pH to 10 with 3 mol/l NaOH, the amount of nitrotyrosine present in the TNM-treated BSA solution was measured at 430 nm (17).

ELISA. A standard curve was constructed by incubating in the wells serial dilutions of nitro-BSA in 0.1 mol/l Na_2CO_3-$NaHCO_3$ coating buffer at a pH of 9.6. Plasma samples assayed for nitrotyrosine were diluted up to five times. Standard and plasma samples were applied to Maxisorp ELISA plates and allowed to bind overnight at 4°C. Afterward, nonspecific binding sites were blocked with 1% BSA in PBS. The wells were incubated at room temperature for 2 h with a mouse IgG monoclonal anti-nitrotyrosine antibody (5 μg/ml) with a peroxidase-conjugated goat anti-mouse IgG secondary antibody diluted 1:4,000. After the plates were washed, the peroxidase reaction product was generated using a tetramethylbenzidine microwell peroxidase substrate. Plates were incubated for 10 min at room temperature, and the reaction was stopped with 50 μl/well of stopping reagent and read at 492 nm in a microplate reader. The concentration of nitrated proteins that inhibited anti-nitrotyrosine antibody binding was estimated from the standard curve and expressed as micromoles per liter of nitrotyrosine (15).

Endothelial function

Endothelial function was evaluated by measuring the FMD of the brachial artery. Vasodilation responses of the brachial arteries were measured by an ultrasound technique by two skillful examiners. The validity of this method has been confirmed in previous studies (18,19). Briefly, the diameter of the brachial artery was measured from B-mode ultrasound images using a 7.5-MHz linear array transducer (SSH-160A; Toshiba, Tokyo, Japan). Flow velocity in the brachial artery was measured using a pulsed Doppler signal at a 70° angle to the vessel, with the range gate (1.5 mm) in the center of the artery. The brachial artery was scanned in the antecubital fossa in a longitudinal fashion. Depth and gain settings were optimized at the beginning of the study and were kept constant throughout the recording period. When a satisfactory transducer position was found, the sur-

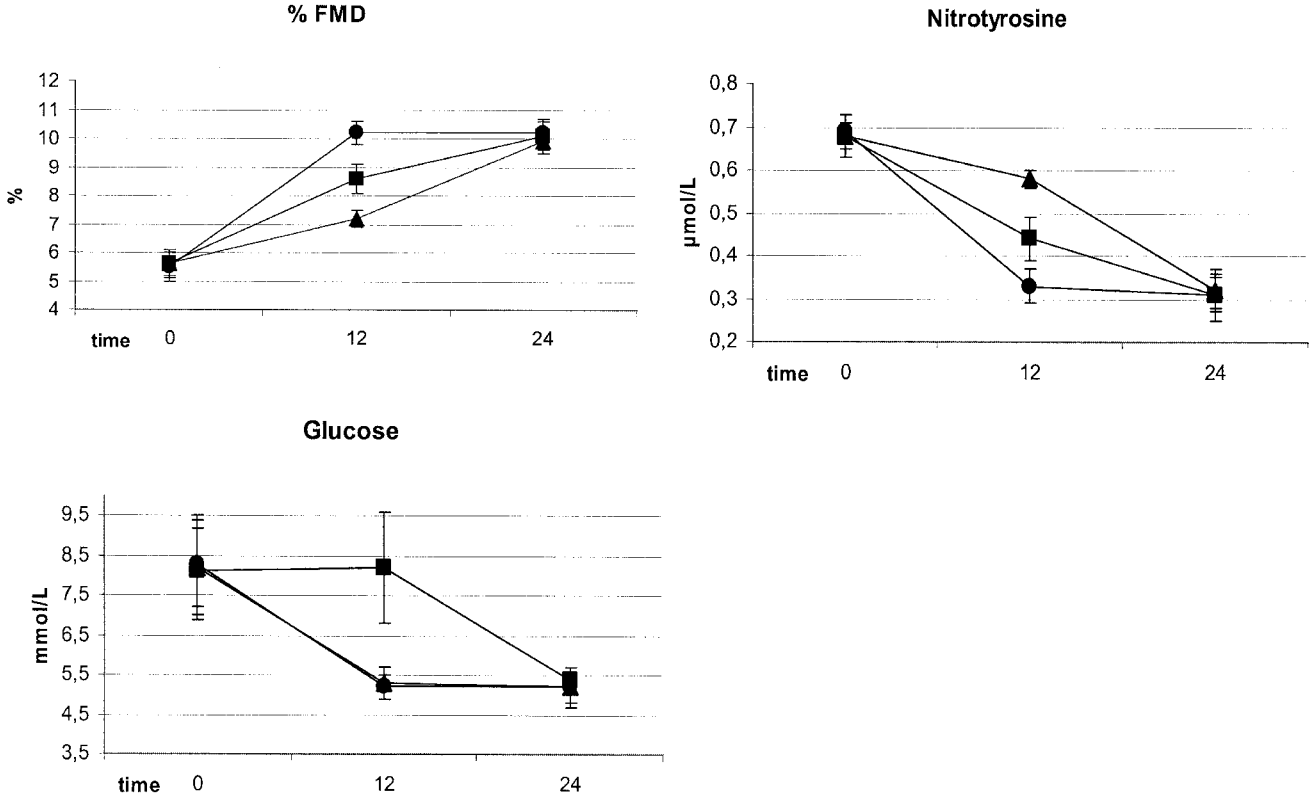

Figure 1—*Glycemia, flow-mediated dilation, and nitrotyrosine plasma levels in the type 1 diabetic patients treated with insulin and vitamin C for 24 h ●, vitamin C for 24 h + insulin for 12 h ■, and insulin for 24 h + vitamin C for 12 h ▲.*

face of the skin was marked, and the arm remained in the same position throughout the study. Each subject lay quietly for 10 min before the first scan.

At the end of each test, the subjects lay quietly for 15 min. Then, sublingual nitroglycerin (0.3 mg) was administered, and 3 min later the last measurements were obtained. The response to nitroglycerin was used as a measure of endothelium-independent vasodilation. All studies were performed in a quiet and temperature-controlled room (22–23°C).

After baseline measurements of the diameter and flow velocity in the brachial artery, a blood pressure cuff placed around the forearm was inflated with a pressure of 250–300 mmHg and was released after 5 min. Diameter and flow velocity were continuously measured from cuff inflation to due time after cuff deflation. The ultrasound images were recorded on a super VHS videocassette recorder (BR-S601M; Victor, Isesaki, Japan), and the arterial diameter was measured at a fixed distance from an anatomical marker with ultrasonic calipers by two independent observers. Measurements were taken from the anterior to the posterior interface between the media and adventitia ("m" line) at the end of diastole, coincident with the R wave on a continuously recorded electrocardiogram (18). The diameters of four cardiac cycles were analyzed for each scan, and the measurements were averaged. Diameter measurements for the reactive hyperemia were taken 45–90 s after cuff deflation to measure peak diameter (18). Responses of the vessel diameters to the reactive hyperemia and nitroglycerin were expressed as the percent increase above the baseline value of the diameter. Blood flow was calculated by multiplying the velocity-time integral of the Doppler flow signal by heart rate and the vessel cross-sectional area. The increase in the blood flow was calculated by dividing the maximum flow within the first 15 s after the cuff deflation by the flow at baseline (18).

In our study, the interobserver variability for the repeated measurements of resting arterial diameter was 0.04 ± 0.02 mm. The intraobserver variability for the repeated measurements of resting arterial diameter was 0.02 ± 0.02 mm.

Statistical analysis

As in previous studies (6,20), the Kolmogorov-Smirnov algorithm was used to determine whether each variable had a normal distribution. Comparisons of baseline data among the groups were performed using unpaired Student's *t* tests. The changes in variables during the tests were assessed by two-way ANOVA with repeated measures. If differences reached statistical significance, post hoc analyses with two-tailed paired *t* tests were used to assess differences at individual time periods in the study, using Bonferroni correction for multiple comparisons. Statistical significance was defined as $P < 0.05$.

RESULTS — Baseline nitrotyrosine was increased in diabetic patients, whereas FMD was reduced (Table 1). With treatments A and C, glycemia was almost normalized after 12 and 24 h ($P < 0.001$ vs. baseline) (Fig. 1), whereas with treatment B, glycemia was normal at 24 h. After 12 h, nitrotyrosine plasma levels decreased significantly with all the treatments ($P < 0.01$ vs. baseline) (Fig. 1): more significantly with treatment C compared with the other two ($P < 0.01$ vs. A and $P < 0.05$ vs. B, respectively) and more with treatment B than with A ($P < 0.05$) (Fig. 1). At 12 h, FMD increased ($P < 0.01$ vs. baseline) (Fig. 1): again more significantly with treatment C than with the other two ($P < 0.01$ vs. A

and $P < 0.05$ vs. B, respectively) and more with treatment B than with A ($P < 0.05$) (Fig. 1).

After 24 h, the plasma levels of nitrotyrosine were significantly decreased compared with the levels of 12 h with treatments A and B ($P < 0.01$) but not with treatment C. After 24 h, there were no differences between the plasma levels of nitrotyrosine reached with each of the three treatments.

FMD was significantly increased compared with the levels after 12 h with treatments A and B ($P < 0.01$) but not with C. Also after 24 h, for FMD there were no differences between the three treatments. Endothelium-independent vasodilation did not change during any of the tests (data not shown).

CONCLUSIONS — This study confirms that even with normalization of glycemia, endothelial dysfunction persists in type 1 diabetic patients (8,9). At the same time, the possibility of improving endothelial function in type 1 diabetes using an antioxidant, in particular vitamin C, is also confirmed (21). However, for the first time, we are able to show that with the combination of vitamin C and the normalization of glycemia, endothelial function can be almost normalized in type 1 diabetic patients.

The role of oxidative stress in this phenomenon appears crucial. When endothelial function is still altered after 12 h of normalization of glycemia or 12 h of vitamin C treatment, nitrotyrosine, a good marker of peroxynitrite and nitrosative stress, is still increased, whereas when endothelial function is normalized, with the combination of glycemia control and vitamin C, nitrotyrosine is also normalized.

Our data confirm that hyperglycemia induces endothelial dysfunction through the generation of oxidative stress, because the administration of vitamin C (in protocol B) in the presence of hyperglycemia restores endothelial function and reduces the nitrotyrosine plasma level and because normalization of glycemia (in protocol A) is accompanied by an improvement of both endothelial function and nitrotyrosine. In the case of protocol A, a possible role for insulin, more than for the reduction of hyperglycemia, in determining the improvement of endothelial dysfunction might be supposed. However, very recently, Ellger et al. (22) have shown, in an animal model, that it is the reduction of glucose toxicity more than the action of insulin that improves endothelial function.

As reported above, previous studies have shown that in type 1 diabetic patients, even with normalizing of glycemia, endothelial dysfunction still persists (8,9), which suggests that long-lasting hyperglycemia can induce permanent alterations in endothelial cells, leading to permanent endothelial dysfunction. Our data suggest that these permanent alterations induce persistence of endothelial dysfunction through the generation of oxidative stress, because by adding vitamin C to insulin treatment, endothelial function is almost normalized. Interestingly, these in vivo data are consistent with preliminary in vitro results showing that in endothelial cells in culture, overproduction of free radicals persists even after normalization of glucose and is accompanied by a prolongation of the induction of protein kinase C (PKC)-β, NAD(P)H oxidase, Bax, collagen, and fibronectin, in addition to nitrotyrosine (23), which suggests that oxidative stress may be involved in this effect.

The finding that only the simultaneous control of glycemia and oxidative stress can normalize endothelial function in type 1 diabetic patients is clearly relevant. This evidence seems to suggest the existence of two different pathways working in the generation of endothelial dysfunction in type 1 diabetes: one directly related to hyperglycemia and one not. The explanation of this second pathway may be, at the moment, only speculative. Brownlee (11) has recently pointed to the role of hyperglycemia-induced overproduction of superoxide by the mitochondrial electron transport chain in triggering several pathways of injury involved in the pathogenesis of diabetic complications (PKC, hexosamine and polyol pathway fluxes, and advanced glycation end product [AGE] formation) by inhibiting glyceraldehyde-3-phosphate dehydrogenase activity (24). Hyperglycemia also favors, through the activation of nuclear factor-κB, an increase in the expression of both NAD(P)H and of inducible nitric oxide synthase (NOS) (25), which would be expected to result in an excess of both nitric oxide (NO) and $\cdot O_2^-$. NO is thought to contribute to endothelial dysfunction in several different ways. First, $\cdot O_2^-$ may also directly react with and quench NO, thereby reducing the efficacy of a potent endothelium-derived vasodilator system that participates in the general homeostasis of the vasculature (26), and evidence suggests that during hyperglycemia reduced NO availability exists (5). Second, as mentioned above, $\cdot O_2^-$ overproduction when accompanied by increased NO generation favors the formation of the strong oxidant $ONOO^-$ (27), and overproduction of both $\cdot O_2^-$ and NO has been reported in response to hyperglycemia (28,29). Finally, in diabetes, the endothelial form of NOS is upregulated but is very sensitive to free radicals generated during hyperglycemia (30). In this situation, NOS synthesizes the superoxide anion instead of NO, leading to oxidative and nitrosative stress (30). It has been shown that a stable protein adduct, nitrotyrosine, is a marker of $ONOO^-$ (31) and $\cdot NO_2$ (32) (for an extensive review, see ref. 10). Moreover, increased oxidative and nitrosative stress activates the nuclear enzyme, poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 activation, on the one hand, depletes its substrate, NAD^+, slowing the rate of glycolysis, electron transport, and ATP formation. On the other hand, it inhibits glyceraldehyde-3-phosphate dehydrogenase by poly(ADP-ribosy)lation. These processes result in acute endothelial dysfunction in diabetic blood vessels, which contributes importantly to the development of various diabetes complications. Accordingly, hyperglycemia-induced activation of PKC isoforms, hexosaminase pathway flux, and AGE formation is prevented by blocking of PARP-1 activity (for an extensive review, see refs. 33,34). However, it is well recognized that chronic hyperglycemia induces the formation of AGEs, and chronic hyperglycemia is thought to alter mitochondrial function through glycation of mitochondrial proteins (35). This premise is important because a recent study, for the first time, has described a direct relationship between the formation of intracellular AGEs on mitochondrial proteins, the decline in mitochondrial function, and the excess formation of reactive species (36): mitochondrial respiratory chain proteins that underwent glycation were prone to produce more $\cdot O_2^-$, independently from the level of hyperglycemia. Therefore, a possible explanation for the previous evidence and for our data is that two pathways are simultaneously working: one due to the actual level of glycemia and another one to the long-lasting damage induced in endothelial cells by chronic hyperglycemia, possibly through nonenzymatic glycation of mitochondria.

It is also surprising that vitamin C

alone cannot normalize endothelial function if oxidative stress is the key convergent effector of both hyperglycemia and of the long-lasting damage (nonenzymatic glycation of mitochondria?). However, it may be hypothesized that when hyperglycemia is present, it induces free radical generation, which can only be partly counterbalanced by the antioxidant treatment: the persistence of increased levels of nitrotyrosine in protocol B supports this hypothesis. Conversely, when hyperglycemia is normalized, the possible presence of a second pathway, still producing free radicals, also may explain the incomplete action of vitamin C.

In any case, although the molecular basis for our findings is not clear, we believe that the clinical impact of our findings is important. First, the persistence of endothelial dysfunction, which is a strong predictor of a cardiovascular event (4), even when glycemia is normalized, may help to explain the recent results from the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications study, which showed, for the first time, the influence of the early glycemic control on the progression to macrovascular events (3). Also of relevance is the evidence that in our study nitrotyrosine is still elevated even in the presence of near-normal glycemia, because nitrotyrosine has been shown to be an independent predictor of cardiovascular disease (37). Furthermore, our data seem to confirm that early continuous aggressive treatment of glycemia is important to avoid future complications. Incidentally, in our opinion, the recent findings showing the existence of a continuum between the values of glycemia, endothelial dysfunction, and the risk of a cardiovascular event, even in nondiabetic patients, supports this concept (38,39).

In summary, our study shows, for the first time, that normalization of both endothelial dysfunction and oxidative stress can be achieved in type 1 diabetic patients, with a combination of near-normalization of glycemia and antioxidant treatment. Future long-term controlled trials may help in understanding the possible clinical impact of this finding on the prognosis of cardiovascular disease in type 1 diabetic patients.

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