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Impact of Oxidative Stress on the Endothelial Dysfunction of Children and Adolescents With Type 1 Diabetes Mellitus: Protection by Superoxide Dismutase?

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ABSTRACT: Diabetes mellitus is associated with endothelial dysfunction and oxidative stress (OS). We investigated whether these abnormalities are interrelated in children and adolescents with type 1 diabetes mellitus (T1DM) and if early OS markers predictive of vascular dysfunction can be identified. Thirty-five T1DM patients were matched for sex, age, height, and weight with nondiabetic subjects as healthy controls (CO). Flow-mediated dilatation (FMD), carotid intima media thickness (IMT), and OS status in fasting blood were measured. Diabetic children had impaired FMD (6.68 ± 1.98 *versus* 7.92 ± 1.60% in CO, $p = 0.004$), which was more pronounced in boys. The degree of FMD impairment was not related to the lower plasma levels of antioxidants or to the higher glucose, glycation, lipids, and peroxidation products. Erythrocyte superoxide dismutase activity, copper/zinc superoxide dismutase (Cu/Zn SOD), was higher in diabetic subjects (1008 ± 224 *versus* 845 ± 195 U/g Hb in CO, $p = 0.003$) and was positively associated with FMD. After correcting for diabetes and gender, the subgroup of children with high Cu/Zn SOD (>955 U/g Hb) had a significantly better FMD ($p = 0.035$). These results suggest that higher circulating Cu/Zn SOD could protect T1DM children and adolescents against endothelial dysfunction. Low Cu/Zn SOD is a potential early marker of susceptibility to diabetic vascular disease. **(*Pediatr Res* 62: 456–461, 2007)**

Diabetes mellitus is an important risk factor for atherosclerosis and both the incidence and mortality of cardiovascular disease are increased in diabetic patients (1). Among the various pathophysiological mechanisms mediating the atherosclerotic process, both oxidative stress (OS) and endothelial dysfunction occur at an early stage in animal models of diabetes (2).

Oxidative stress is defined as the change in the pro-oxidant/antioxidant balance in favor of the former, potentially leading to biologic damage to macromolecules and cell dysfunction (3). As a result of hyperglycemia, excessive pro-oxidants (free radicals and reactive oxygen species) are formed *via* autooxidation of glucose, nonenzymatic glycation and formation of advanced glycation end products, increased flux through the polyol and hexosamine pathways, and activation of protein kinase C. These processes also lead to decreased antioxidant defenses. Brownlee (4) has linked all these abnormalities to the excessive production of superoxide by the mitochondria.

In children with type 1 diabetes mellitus (T1DM), increased OS has been reported to be present even shortly after diagnosis (5). Other reports showed the parallelism between OS and abnormal markers of endothelial cell function (such as E-Selectin and ICAM-1) in young T1DM patients, suggesting a link between these two abnormalities (6). Ultrasound testing of skin microcirculation and of brachial artery flow-mediated dilatation (FMD) have demonstrated early endothelial dysfunction in diabetic children and adolescents (7).

Since it has been shown that foam cell accumulation in the vascular wall is already present in 69% of adolescents in the general population (8), it can be postulated that the diabetes-induced endothelial abnormalities might be directly related to an increase in intima media thickness and thus be involved in the early pathogenesis of vascular dysfunction that underlies the increased atherosclerotic risk in diabetes. The mechanisms mediating or modulating these possible relationships have not been fully identified yet. In the present study, we investigated the relationship between endothelial function, carotid intima media thickness, and the oxidant-antioxidant balance. The ultimate aim is to investigate whether markers of OS can help to identify the diabetic children who are more susceptible to develop diabetic vascular disease.

MATERIALS AND METHODS

Study subjects and design. Diabetic children and adolescents regularly attending the Diabetes Outpatient Clinic of the Antwerp University Hospital were recruited consecutively. All patients were treated with a basal-bolus insulin regimen with ≥4 subcutaneous injections daily. Exclusion criteria were presence of other diseases, regular medications other than insulin, urinary albumin excretion exceeding 15 μg/min in an overnight timed urine collection, neuropathy or proliferative retinopathy. A nondiabetic control group was recruited among the children or friends of the hospital staff members or of the diabetic children. In view of the influence of age, gender, height, and weight on the cardiovascular function parameters, great care was taken to carefully match all diabetic subjects and controls for these parameters. Pubertal stage classification was based on the determination of circulat-

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Abbreviations: CO, healthy control; **Cu/Zn SOD,** copper/zinc superoxide dismutase; **DM,** diabetic groups;; **d-ROM,** determinable reactive oxygen metabolites; **FMD,** flow-mediated dilatation; **IMT,** intima media thickness; **MDA,** malondialdehyde; **OS,** oxidative stress; **T1DM,** type 1 diabetes mellitus

ing levels of hormones, clinical examination, and age. All study subjects were on an average standard Flemish school-child normocaloric diet, which did not differ in the diabetic group. There were no vegetarians and they did not receive any supplements of vitamins or antioxidants. Similarly, physical activity was that of an average school-going population (ranging from 2 to 12 h of sport per week).

Out of 81 diabetic and nondiabetic children initially recruited for the study, 7 diabetic children were not included in the final statistical analysis because of celiac disease, obesity, hypercholesterolemia, maturity-onset diabetes of the young (MODY), and lack of a matched control. Two controls were not included because of bronchitis and use of steroid medication at the time of the study.

The study was approved by the Hospital Ethics Committee (Comité voor Medische Ethiek, Universitair Ziekenhuis Antwerpen, Wilrijkstraat 10, approval number 3/29/101) and all participants or their parents signed an informed consent form. On the day of the study, a fasting blood sample was obtained and FMD and carotid intima media thickness (IMT) were measured.

Routine blood tests including glucose, glycated Hb (HbA_{1c}), lipid profile and routine blood count, and biochemistry were analyzed in the laboratory of the Antwerp University Hospital. Total plasma homocysteine was assayed by HPLC (Bio Rad kit 195-4075, Hercules, Ca) (coefficient of variation CV 3.4%).

OS status was evaluated by measuring blood concentrations of individual antioxidants, global plasma antioxidant capacity, and products of lipid peroxidation as recently described in detail (9). In short, alpha-tocopherol, retinol, and ascorbate were measured by reversed phase HPLC. Glutathione (GSH) in whole blood and protein thiols in plasma were measured by a colorimetric method using Ellman's reagent. Total plasma antioxidant capacity was evaluated by inhibition of peroxyl-induced chemiluminescence (TAC-PI) and by radical scavenging capacity, expressed as Trolox equivalents (TAC-TE). Plasma d-ROM (determinable reactive oxygen metabolites) was measured using a commercial kit (Pharmalab d-ROMs, Parma, Italy) and expressed as tert-butyl hydroperoxide equivalents. Plasma malondialdehyde (MDA) was analyzed by reverse phase HPLC of the adduct formed by reaction with thiobarbituric acid. Copper/zinc superoxide dismutase (Cu/Zn SOD) and glutathione peroxidase (GPX) in erythrocyte hemolysate were assayed using commercial kits (RANSOD and RANSEL respectively, Randox, Crumlin, UK, CV 14.5% and 5.9%).

FMD was evaluated by ultrasonography of the right brachial artery at rest (baseline brachialis diameter, BBD) and during reactive hyperemia after inflating and deflating a forearm blood pressure cuff (200 mm Hg or at least 50 mm Hg above peak systolic blood pressure for 4 min). Continuous ECG registration was used to measure the correct end-diastolic diameter, coincident with the R-wave. Postocclusion measurements were taken every 30 s over the following 240 s. To calculate the maximal dilatation compared with baseline (peak FMD %), the mean of three measurements at baseline, and the maximal postocclusion value were used.

Carotid IMT was evaluated on patients in the supine position, with the head turned 45° away from the side being scanned. The reference point for measurement was the beginning of the dilatation of the carotid bulb. The two-dimensional B-mode image of the posterior wall of the right common carotid artery was gained 1 to 2 cm proximal to the carotid bifurcation. The radiofrequency signals originating from an M-line perpendicular to the longitudinal and transversal axes of the artery, R-wave triggered, were analyzed three times using three different interrogation angles: 0° from midline, 30–60° from midline (anterior oblique), 90–100° from midline (lateral). Mean IMT of the three measurements was calculated.

Ultrasound studies were performed using the AU5 Ultrasound system (Esaote, Biomedica, Genova, Italy), equipped with a 10 Mhz linear-array transducer. Data analysis was performed using the Wall-Tracker System (WTS, P-Medical, Maastricht, The Netherlands).

Statistical analysis. Results were expressed as mean ± SD or as geometric mean (95% confidence intervals) for data not compatible with a Gaussian distribution. Data were analyzed using the statistical package SPSS Version 11.0 (Chicago, IL). Differences between groups were calculated using two-factor ANOVA to test for the independent effect of diabetes and of gender as well as the interaction between both factors. Non-Gaussian data were log-transformed before application of the parametric tests. Multiple linear regression (stepwise) and analysis of covariance were applied to identify the relationship between the various parameters. Two-tailed p values ≤ 0.05 (or adjusted for multiple comparisons according to Bonferroni) were considered as statistically significant. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

With regard to clinical characteristics (Table 1), the healthy control (CO) and diabetic groups (DM) did not differ in age, gender distribution, pubertal stage, weight, and height. Serum cholesterol was higher in DM (4.71 ± 0.89 *versus* 4.07 ± 0.61 mM, $p = 0.001$ for the independent effect of diabetes). Likewise, serum triacylglycerols were higher in DM (1.09 ± 0.47 *versus* 0.79 ± 0.41 mM, $p = 0.006$).

Table 1. *General clinical and metabolic characteristics of the study subjects*

	Healthy controls		Diabetes mellitus		P-value ANOVA		
Characteristic	Girls (n = 19)	Boys (n = 18)	Girls (n = 19)	Boys (n = 16)	Diabetes	Gender	Interaction
Age (y)	14.5 ± 2.7	14.4 ± 3.3	14.5 ± 2.6	14.2 ± 3.6	NS	NS	NS
Pre-/pubertal/post-pubertal (0/1/2)§	3/10/6	6/6/6	2/9/8	8/3/5	NS	0.029	
Duration of diabetes (y)	Not relevant	Not relevant	6.2 ± 4.0	7.0 ± 3.1		NS	
Fasting glycemia (mg/dL)	81 ± 6	83 ± 6	222 ± 86	235 ± 86	<0.0005	NS	NS
HbA_{1c} on day of study (%)	5.16 ± 0.17	5.28 ± 0.15	9.11 ± 1.56	8.36 ± 1.50	<0.0005	NS	NS
Insulin dose (U/Kg/d)†	Not relevant	Not relevant	1.08 ± 0.44	0.98 ± 0.24		NS	
Weight (kg)	52.1 ± 11.4	51.7 ± 16.2	51.9 ± 12.5	50.3 ± 17.5	NS	NS	NS
Height (cm)	161 ± 11	165 ± 18	159 ± 14	163 ± 16	NS	NS	NS
BMI (kg/m^2)	19.8 ± 2.6	17.4 ± 2.1	20.2 ± 2.5	18.4 ± 2.6	NS	0.008	NS
Blood pressure systolic (mm Hg)	115 ± 10	118 ± 11	114 ± 15	115 ± 13	NS	NS	NS
Diastolic (mm Hg)	64 ± 6	62 ± 6	65 ± 14	64 ± 6	NS	NS	NS
Flow mediated brachial dilatation (%)	7.91 ± 1.34	7.94 ± 1.86	7.43 ± 2.03	5.89 ± 1.62	0.004	0.074	0.064
Carotid intima media thickness (mm)	0.482 ± 0.041	0.468 ± 0.047	0.497 ± 0.058	0.493 ± 0.069	NS	NS	NS
Fasting serum total cholesterol (mmol/L)	4.13 ± 0.70	4.00 ± 0.51	5.01 ± 0.83	4.38 ± 0.86	0.001	0.032	NS
Fasting serum HDL cholesterol (mmol/L)	1.59 ± 0.30	1.63 ± 0.40	1.70 ± 0.32	1.76 ± 0.39	NS	NS	NS
Fasting serum LDL cholesterol (mmol/L)	2.17 ± 0.66	2.02 ± 0.49	2.74 ± 0.70	2.21 ± 0.71	0.016	0.028	NS
Fasting serum triacylglycerol (mmol/L)	0.82 ± 0.39	0.76 ± 0.43	1.25 ± 0.52	0.92 ± 0.35	0.006	0.059	NS
Serum iron (μmol/L)	19.3 ± 7.3	16.9 ± 12.0	18.9 ± 7.0	16.3 ± 6.6	NS	NS	NS
Serum TIBC (μmol/L)	66.6 ± 13.0	69.3 ± 5.1	72.7 ± 10.3	62.4 ± 7.0	NS	0.095	0.006
Hemoglobin (g/dL)	14.4 ± 1.6	14.6 ± 1.3	14.6 ± 2.1	14.1 ± 1.3	NS	NS	NS

Values are expressed as means ± SD, number of observations. § Pubertal stage was assessed by clinical and hormonal determination. † Six children received insulin by pump. The p value was obtained by χ^2 and by two-factor ANOVA to determine the independent effect of diabetes or gender and the interaction between these two factors.

Table 2. *Oxidative stress status of the study subjects*

Characteristic	Healthy controls		Diabetes mellitus		*p* value ANOVA		
	Girls (n = 19)	Boys (n = 18)	Girls (n = 19)	Boys (n = 16)	Diabetes	Gender	Interaction
Plasma TAC-TE (μmol/L)	179 ± 28.4	198 ± 39.0	149 ± 20.5	160 ± 27.2	<0.0005	0.047	NS
Plasma TAC-PI (% inhibition)	69.4 ± 11.5	69.2 ± 10.3	71.7 ± 5.8	69.9 ± 4.5	NS	NS	NS
Erythrocyte SOD (U/g Hb)	869 ± 203	824 ± 190	979 ± 160	1039 ± 280	0.003	NS	NS
Erythrocyte GPX (U/g Hb)	44.8 ± 11.2	42.1 ± 9.3	46.8 ± 10.3	40.0 ± 7.9	NS	0.054	NS
Serum albumin (g/L)	45.1 ± 2.4	45.3 ± 2.4	39.6 ± 3.0	40.9 ± 3.4	<0.0005	NS	NS
Serum proteins (g/L)	75.7 ± 2.6	75.6 ± 4.8	72.6 ± 6.2	71.1 ± 7.9	0.017	NS	NS
Serum total bilirubin (μmol/L)	11.9 ± 7.9	9.8 ± 6.0	7.3 ± 2.5	10 ± 6.9	NS	NS	NS
Plasma protein thiols (μmol/g protein)	5.22 ± 1.79	5.28 ± 1.86	4.37 ± 1.65	4.08 ± 1.79	0.025	NS	NS
Blood glutathione (μmol/g Hb)	6.09 ± 1.05	5.76 ± 1.13	5.44 ± 1.01	5.62 ± 0.95	NS	NS	NS
Serum uric acid (μmol/L)	241 ± 47	258 ± 77	189 ± 35	220 ± 33	<0.0005	0.047	NS
Plasma ascorbate (μmol/L)	78.4 ± 26.3	85.4 ± 29.2	71.7 ± 17.9	62.8 ± 18.3	0.016	NS	NS
Serum retinol (μmol/L)	3.54 ± 1.13	3.42 ± 1.18	4.02 ± 1.15	3.18 ± 1.39	NS	NS	NS
Serum α-tocopherol (μmol/L)	23.0 ± 5.8	21.13 ± 5.2	22.8 ± 5.9	23.68 ± 11.35	NS	NS	NS
(μmol/mmol total lipid)	4.77 ± 1.48	4.57 ± 1.17	3.66 ± 0.77	4.40 ± 1.44	0.054	NS	NS
Plasma dROM (mmol/L TBOOH)	2.46 ± 1.06	2.27 ± 0.57	3.71 ± 1.31	2.66 ± 0.75	0.001	0.012	0.082
Plasma malondialdehyde (μmol/L)	0.495 ± 0.122	0.460 ± 0.140	0.619 ± 0.146	0.626 ± 0.219	<0.0005	NS	NS
(μmol/mmol total lipid)	0.104 ± 0.039	0.099 ± 0.035	0.103 ± 0.019	0.115 ± 0.032	NS	NS	NS
Serum ferritin (pmol/L)‡	63 (50–80)	60 (47–76)	74 (59–94)	94 (73–122)	0.012	NS	NS
Plasma myeloperoxidase (U/mL)	21.8 ± 14.0	28.3 ± 28.0	39.6 ± 73.1	20.1 ± 6.4	NS	NS	NS
Homocystein (μmol/L)	7.31 ± 2.21	7.87 ± 1.92	6.33 ± 1.83	6.82 ± 1.17	0.055	NS	NS

Values are expressed as means ± SD or ‡ as geometric mean (95% confidence interval) obtained after log transformation of the non-Gaussian distributed data. The *p* value was obtained by two-factor ANOVA to determine the independent effect of diabetes or gender and the interaction between these two factors. TAC-TE denotes total antioxidant capacity expressed as Trolox equivalents and TAC-PI as percentage inhibition of chemiluminescence. SOD and GPX denote superoxide dismutase and glutathione peroxidase, respectively; dROM denotes determinable reactive oxygen metabolites as a measure of peroxides and is expressed as tert-butyl hydroperoxide (TBOOH) equivalents.

OS status was assessed by measuring antioxidant concentrations, antioxidant enzyme activities, body iron stores, and products of peroxidation (Table 2). Independently of gender, diabetic children had lower levels of plasma antioxidant capacity (TAC-TE), which was accompanied by lower levels of hydrophilic antioxidants (uric acid and ascorbate) and lower serum proteins and albumin in particular. Likewise, the thiol content in plasma proteins was lower in DM (4.22 ± 1.69 *versus* 5.25 ± 1.80 μmol/g protein, $p = 0.025$). Serum retinol did not differ and α-tocopherol tended to be lower in DM only when expressed relative to serum lipids (3.97 ± 1.14 *versus* 4.67 ± 1.32 μmol/mmol cholesterol + triacylglycerols in CO, $p = 0.054$). In contrast to no differences in glutathione peroxidase (GPX), superoxide dismutase (Cu/Zn SOD) was higher in DM (1008 ± 224 *versus* 845 ± 195 U/g Hb, $p = 0.003$). Plasma lipid peroxidation products in the form of d-ROM and MDA were higher in DM (0.622 ± 0.176 *versus* 0.478 ± 0.130 μM, $p < 0.0005$) but this difference disappeared when expressing MDA relative to cholesterol or to total lipids (cholesterol + triacylglycerols) in serum. In the DM group, glycemic control monitored as HbA_{1c} was negatively related to the endogenous circulating antioxidants uric acid ($r = -0.55$, $p = 0.007$), glutathione, ($r = -0.38$, $p = 0.038$) and bilirubin ($r = -0.49$ $p = 0.017$) and positively to d-ROM ($r = 0.39$ $p = 0.039$). These relationships were not observed in the CO.

Endothelial function as assessed by brachial artery FMD, was significantly impaired in DM (6.68 ± 1.98 *versus* 7.92 ± 1.60%, $p = 0.004$ for the independent effect of DM) and this impairment tended to be more pronounced in boys ($p = 0.06$ for the interaction DM and gender). Carotid intima media thickness (0.495 ± 0.063 in DM *versus* 0.481 ± 0.052 mm in CO, $p = 0.14$) and blood pressure were not significantly different in diabetes and were not affected by gender. It should be noted, however, that the power to detect differences in these parameters only amounted to <0.31 in this study.

Analysis of the factors influencing FMD revealed, respectively, a positive and negative relationship with serum HDL cholesterol and triacylglycerols in CO but these relationships were lost in DM (Fig. 1, *A* and *B*). In contrast, there was no significant correlation with LDL cholesterol (Fig. 1*C*) or with parameters of glycemic control (fasting plasma glucose or HbA1c) in either group. In the diabetic group but not in the CO, FMD was negatively related to carotid IMT (Fig. 1*D*). Moreover, IMT was strongly inter-related with daily insulin dose (units/kg) in the DM ($r = 0.46$, $p = 0.007$). A multiple regression model including these variables shows that the variance of FMD is explained by triacylglycerols (24%) in CO and by IMT (or insulin dose) in DM (18%). In the whole study population or in either group separately, neither FMD nor carotid IMT were significantly related to levels of circulating nonenzymatic antioxidants or peroxidation products. The sole marker of OS status, which was related to FMD, was Cu/Zn SOD but only in DM (Fig. 2*A*). Comparison of the groups with Cu/Zn SOD values higher or lower than 955 U SOD/g Hb (median of the whole population) revealed that the high Cu/Zn SOD group had better FMD in both girls and boys of the DM and the CO groups ($p = 0.035$ for the independent effect of high Cu/Zn SOD after correcting for the effect of diabetes and gender) (Fig. 2*B*). Diabetic girls had normal

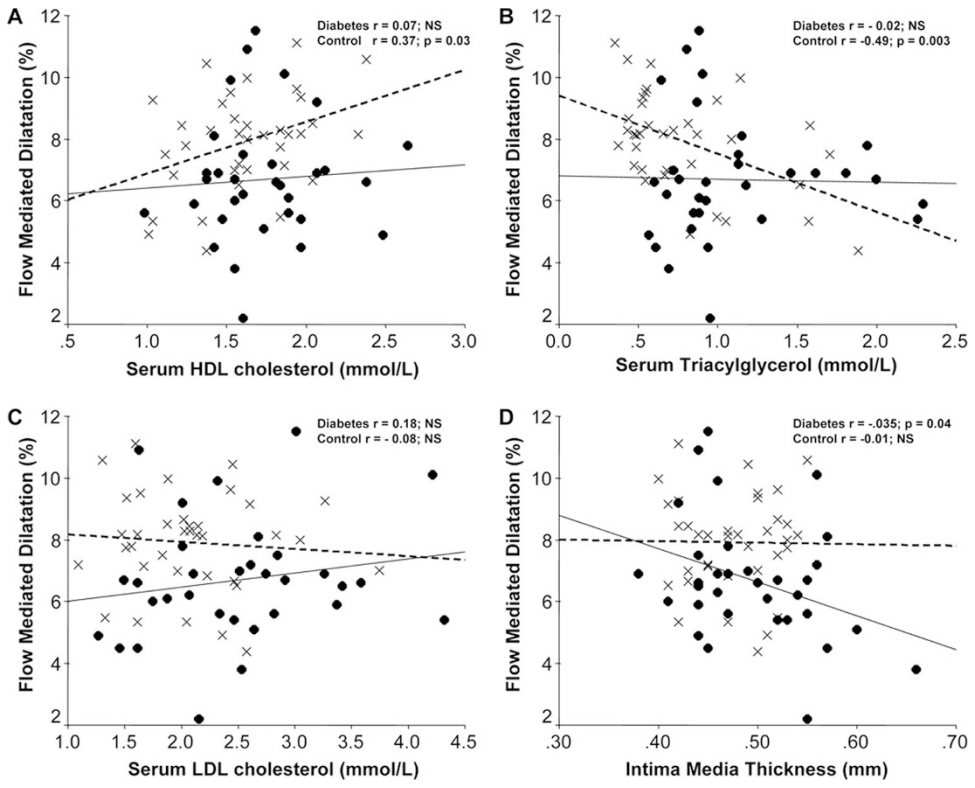

Figure 1. Scatter plots of the relationship between flow mediated vasodilatation and serum HDL cholesterol (*A*), triacylglycerol (*B*), LDL cholesterol (*C*), and carotid intima media thickness (*D*) in diabetic (● and continuous line) and nondiabetic controls (X and discontinuous line).

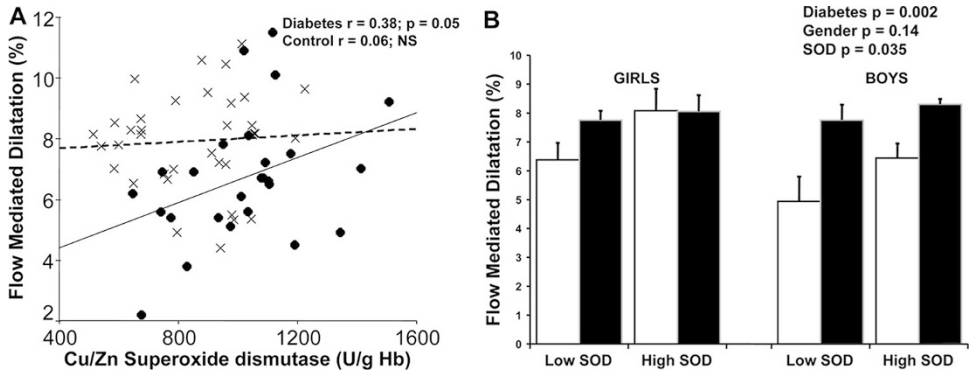

Figure 2. (*A*) Scatter plot of the relationship between flow mediated vasodilatation and erythrocyte Cu/Zn superoxide dismutase activity in diabetic (● and continuous line) and nondiabetic controls (X and discontinuous line). (*B*) Histogram showing flow mediated dilatation in the subgroups divided according to diabetes, gender, and level of erythrocyte Cu/Zn SOD. Shown are means + SEM of diabetic patients (□) and control subjects (■). High and low Cu/Zn SOD refers to the subgroups having Cu/Zn SOD levels respectively ≥ and <955 units/g Hb, which corresponds to the median of the whole study population. Three-factor analysis of variance detects an independent effect of diabetes ($p = 0.002$) and SOD level ($p = 0.035$) but not of gender ($p = 0.14$). The interaction between the factors was: diabetes × gender ($p = 0.08$), diabetes × SOD level ($p = 0.21$), and gender × SOD level ($p = 0.98$).

FMD values when their Cu/Zn SOD activity was above the median (8.08 ± 2.29 *versus* 6.38 ± 1.18% in the low Cu/Zn SOD subgroup). In diabetic boys, the FMD was also better in the high Cu/Zn SOD subgroup (6.44 ± 1.43% *versus* 4.94 ± 1.92% in the low Cu/Zn SOD subgroup) but did not reach the levels seen in control boys (Table 2).

Pubertal stage was not associated with differences in FMD, IMT, Cu/Zn SOD, insulin dose, or OS parameters. Indeed, the lower FMD in diabetic children ($p = 0.004$ for the independent effect of diabetes) persisted after correcting for the confounding effect of pubertal stage with and without that of gender (using three- and two-factor ANOVA). However, the power to detect an independent or interactive effect of pubertal stage only amounted to 0.25 in this study.

DISCUSSION

In this study, we describe OS status in young and adolescent type 1 diabetic patients and its relationship with endothelial function and carotid intima media thickness. Enhanced OS in this group of diabetic patients was illustrated by the increase in plasma peroxidation products (d-ROM and MDA), the decrease in antioxidant molecules (ascorbic acid, urate, and tocopherol relative to lipids) and the decrease in the antioxidant capacity of proteins (thiol content) and of plasma globally (TAC). The negative relationship between endogenous scavenger antioxidants (uric acid, glutathione, and bilirubin) and levels of HbA_{1c} as well as the positive one with peroxidation products points out to the important role of glycemic

control in antioxidant/oxidant balance in these young DM patients. Some antioxidants are probably lost due to renal hyperfiltration in the early phase of T1DM (10). In addition, hyperglycemia leads to redox imbalances and glycation of enzymes and can thus cause impairment in the recycling of molecules such as ascorbate and thiols (11). These findings, together with the increase in Cu/Zn superoxide dismutase, are in accordance with other reports on children (5,6) and on young and adult T1DM patients (12). Enhanced lipid peroxidation *in vivo* has recently been confirmed by detecting increases in urinary F2 isoprostanes which were more marked at onset of the disease and improved in parallel to glycemic control (13).

Diabetes was associated with impairment of FMD that was more outspoken in the diabetic boys. We did not find a correlation between FMD and LDL cholesterol levels in either group, as also seen in older T1DM patients with and without microalbuminuria (14) but in discordance with other studies on younger diabetic children (median 11 y) with a slightly shorter duration of diabetes (4 y) (7). The deleterious effect of triacylglycerols and the protection afforded by HDL cholesterol in the healthy controls were lost in the diabetic group. Taken together, these observations suggest that in this diabetic group of children the impairment of FMD was related to factors other than their worse lipid control.

Despite the fact that our study was not sufficiently powered to detect the diabetes-related differences in IMT which have been reported by Jarvisalo (7), we observed a thicker carotid intima media in the children receiving higher insulin doses (corrected for weight) as also observed in a survey of adult type 1 diabetic patients (15). Both these factors were negatively related to FMD. In contrast, the only factor positively related to FMD in diabetic children was Cu/Zn SOD. We observed that the sub-group of diabetic children with higher circulating erythrocyte Cu/Zn SOD levels had less FMD impairment. In girls, this subgroup had normal FMD function but in boys, the higher Cu/Zn SOD status did not fully counterbalance their more outspoken FMD impairment. These observations suggest that the level of increase in SOD activity seen in diabetic children may be relevant in the defence against the impairment of FMD caused by diabetes.

Since SOD is the enzyme responsible for the neutralization of superoxide and because we did not observe any relationship between FMD and glutathione peroxidase, our data suggest the dominant role of superoxide rather than of peroxides in this diabetic endothelial dysfunction. This idea is supported by the lack of correlations with other markers of OS. The batteries available to monitor OS are still limited and fragmentary and do not allow direct monitoring of superoxide production *in vivo* in human subjects. Nevertheless, multiple observations in animals models have demonstrated an increased production of superoxide in diabetes, especially in the vessel wall (2). Potential mechanisms are the hyperglycemia induced activation of protein kinase C which in turn stimulates NADPH-oxidases to generate superoxide and reactive oxygen species (16). Superoxide interferes with the generation of NO production in several ways such as a decrease in the endothelial nitric oxide synthase (eNOS) expression mediated by activator protein, AP-1 (17), a change in the electrophysiological state of the endothelial cell (18) and the availability of tetrahydrobiopterin, an essential cofactor of eNOS (19). The reaction of superoxide with NO in the vascular wall also contributes to the decreased bioavailability of vascular NO, thus impairing vasodilatory response, as observed in this and other studies.

In several models of diabetes, SOD or SOD-mimetic treatments reverse the impairment of endothelium-dependent relaxation (20–22). It should, however, be stressed that care should be taken when extrapolating conclusions from diabetic animal models to the human situation since, for example, diabetogenic agents such as streptozotocin can influence the gene expression of SOD directly with as a result decreases in SOD activity (23).

Data on the protective role of SOD in human diabetic patients are scarce and may differ depending on the type and duration of diabetes as well as race. Brownlee's unifying theory of complications in diabetes focuses on superoxide release by the mitochondria (4), which would be cleared up by the mitochondrial SOD (the Mn isoform). Our study, however, identified a potential protective role of the cytoplasmic form (Cu/Zn SOD), which is present in the red blood cells. This suggests that the superoxide responsible for the endothelial dysfunction is largely extracellular and present in the lumen of the vessel where it can be neutralized by the red cell Cu/Zn SOD. The relevance of the extracellular form of SOD (ecSOD) in the protection against several phases in the atherosclerotic process is also highlighted by the association of a mutation in the ecSOD gene with prognosis in diabetic hemodialysis patients (24). Indeed, the lower coronary ecSOD activity in adult coronary artery disease patients and its negative correlation to FMD (25) indicate that it plays a key determinant role in the bioavailability of vascular NO (26).

In addition to genetically predetermined levels, up-regulation of SOD synthesis in response to the oxidative injury or glycation caused by hyperglycemia has also been described, again in cell cultures and animal models (27). Interestingly, in T1DM patients with nephropathy, SOD is less up-regulated than in patients without nephropathy, again suggesting that SOD plays an important role in the susceptibility to develop diabetic complications (28).

It is not clear why high Cu/Zn SOD levels are associated with a normal FMD in diabetic girls but not in boys in our study. Apart from the more pronounced impairment in the boys, which would require more intensive counter-regulatory measures, other (hormonal) factors might play a significant role. Apart from the well-established direct receptor-mediated beneficial effects of estrogens on the vascular wall (29), several observations suggest that they can also modulate the vascular oxidant-antioxidant balance (30,31). Our study was not sufficiently powered to study the independent effect of puberty and its influence on the diabetes-induced impairment of FMD. This question needs further investigations aimed at analyzing the relationship between status of specific hormones (for example, also including growth hormone) and endothelial function.

In view of the increasing perception of the serious cardiovascular risk already present in young T1DM patients (32) and

of its rapid progression in young adults (33), early intervention should target the modifiable risk factors in genetically predisposed individuals (with a positive family history of atherosclerosis). Although metabolic control remains the primordial therapeutic target in these patients, it does not explain the total coronary artery disease risk in T1DM (34). In addition to current antismoking campaigns, stimulation of regular physical exercise from an early age, treatment of dyslipidemia and hypertension, our results suggest that targeting OS could possibly help to counteract the early disruption of the delicate redox balance within the vasculature and the endothelial dysfunction caused by diabetes. For example, both vitamin C and E mimic SOD in neutralizing the superoxide production induced by high glucose and have been shown to reduce endothelial dysfunction in DM (35). It has also been shown that in healthy individuals, ascorbate can reverse the acute impairment of vasodilatation caused by hyperglycemia (36). Since our results show that diabetic children have lower plasma ascorbate levels, the promotion of a healthier nutrition based on more fruit intake may be a feasible approach to target this vascular dysfunction in childhood diabetes.

In conclusion, diabetic children have an impaired endothelial function and elevated markers of OS. Those children with a higher erythrocyte Cu/Zn SOD activity have a better endothelium mediated vascular function. Our results suggest that SOD could play a relevant protective role but we acknowledge that an observed association does not demonstrate causality and that the important influence of insulin treatment and possibly resistance requires further investigation. Sufficiently powered prospective and intervention studies are therefore needed to investigate whether levels of erythrocyte Cu/Zn SOD are related to, and can even predict susceptibility to, early endothelial dysfunction and atherosclerosis in young diabetic patients.

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REFERENCES

1. Kannel WB, McGee DL 1979 Diabetes and glucose tolerance as risk factors for cardiovascular disease: the Framingham study. Diabetes Care 2:120–126
2. Tesfamariam B, Cohen RA 1992 Free radicals mediate endothelial cell dysfunction caused by elevated glucose. Am J Physiol 263:H321–H326
3. Sies H 1991 Oxidative stress: from basic research to clinical application. Am J Med. 91:31S–38S
4. Brownlee M 2005 The pathobiology of diabetic complications: a unifying mechanism. Diabetes 54:1615–1625
5. Dominguez C, Ruiz E, Gussinye M, Carrascosa A 1998 Oxidative stress at onset and in early stages of type 1 diabetes in children and adolescents. Diabetes Care 21:1736–1742
6. Elhadd TA, Kennedy G, Hill A, McLaren M, Newton RW, Greene SA, Belch JJ 1999 Abnormal markers of endothelial cell activation and oxidative stress in children, adolescents and young adults with type 1 diabetes with no clinical vascular disease. Diabetes Metab Res Rev 15:405–411
7. Jarvisalo MJ, Raitakari M, Toikka JO, Putto-Laurila A, Rontu R, Laine S, Lehtimaki T, Ronnemaa T, Viikari J, Raitakari OT 2004 Endothelial dysfunction and increased arterial intima-media thickness in children with type 1 diabetes. Circulation 109:1750–1755
8. Stary HC 2000 Lipid and macrophage accumulations in arteries of children and the development of atherosclerosis. Am J Clin Nutr. 72:1297S–1306S
9. Manuel-y-Keenoy B, Van Campenhout A, Aerts P, Vertommen J, Abrams P, Van Gaal L, van Gils C, De Leeuw I 2005 Time course of oxidative stress status in the postprandial and postabsorptive states in type 1 diabetes mellitus: relationship to glucose and lipid changes. J Am Coll Nutr 24:474–485
10. Herman JB, Goldbourt U 1982 Uric acid and diabetes: observations in a population study. Lancet 2:240–243
11. Sinclair AJ, Girling AJ, Gray L, Le Guen C, Lunec J 1991 Disturbed handling of ascorbic acid in diabetic patients with and without microangiopathy during high dose ascorbate supplementation. Diabetologia 34:171–175
12. Dierckx N, Horvath G, van Gils C, Vertommen J, van de Vliet J, De Leeuw I, Manuel-y-Keenoy B 2003 Oxidative stress status in patients with diabetes mellitus: relationship to diet. Eur J Clin Nutr 57:999–1008
13. Davi G, Chiarelli F, Santilli F, Pomilio M, Vigneri S, Falco A, Basili S, Ciabattoni G, Patrono C 2003 Enhanced lipid peroxidation and platelet activation in the early phase of type 1 diabetes mellitus: role of interleukin-6 and disease duration. Circulation 107:3199–3203
14. Dogra G, Rich L, Stanton K, Watts GF 2001 Endothelium-dependent and independent vasodilation studied at normoglycaemia in type I diabetes mellitus with and without microalbuminuria. Diabetologia 44:593–601
15. Muis MJ, Bots ML, Bilo HJ, Hoogma RP, Hoekstra JB, Grobbee DE, Stolk RP 2005 High cumulative insulin exposure: a risk factor of atherosclerosis in type 1 diabetes? Atherosclerosis 181:185–192
16. Channon KM, Guzik TJ 2002 Mechanisms of superoxide production in human blood vessels: relationship to endothelial dysfunction, clinical and genetic risk factors. J Physiol Pharmacol 53:515–524
17. Srinivasan S, Hatley ME, Bolick DT, Palmer LA, Edelstein D, Brownlee M, Hedrick CC 2004 Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. Diabetologia 47:1727–1734
18. Brzezinska AK, Lohr N, Chilian WM 2005 Electrophysiological effects of O2*- on the plasma membrane in vascular endothelial cells. Am J Physiol Heart Circ Physiol 289:H2379–H2386
19. Pannirselvam M, Verma S, Anderson TJ, Triggle CR 2002 Cellular basis of endothelial dysfunction in small mesenteric arteries from spontaneously diabetic (db/db -/-) mice: role of decreased tetrahydrobiopterin bioavailability. Br J Pharmacol 136:255–263
20. Hattori Y, Kawasaki H, Abe K, Kanno M 1991 Superoxide dismutase recovers altered endothelium-dependent relaxation in diabetic rat aorta. Am J Physiol 261:H1086–H1094
21. Schnackenberg CG, Wilcox CS 2001 The SOD mimetic tempol restores vasodilation in afferent arterioles of experimental diabetes. Kidney Int 59:1859–1864
22. Zanetti M, Sato J, Katusic ZS, O'Brien T 2001 Gene transfer of superoxide dismutase isoforms reverses endothelial dysfunction in diabetic rabbit aorta. Am J Physiol Heart Circ Physiol 280:H2516–H2523
23. Kamata K, Kobayashi T 1996 Changes in superoxide dismutase mRNA expression by streptozotocin-induced diabetes. Br J Pharmacol 119:583–589
24. Yamada H, Yamada Y, Adachi T, Fukatsu A, Sakuma M, Futenma A, Kakumu S 2000 Protective role of extracellular superoxide dismutase in hemodialysis patients. Nephron 84:218–223
25. Landmesser U, Merten R, Spiekermann S, Buttner K, Drexler H, Hornig B 2000 Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 101:2264–2270
26. Landmesser U, Hornig B, Drexler H. 2004 Endothelial function: a critical determinant in atherosclerosis? Circulation 109:II27–II33.
27. Weidig P, McMaster D, Bayraktutan U 2004 High glucose mediates pro-oxidant and antioxidant enzyme activities in coronary endothelial cells. Diabetes Obes Metab 6:432–441
28. Hodgkinson AD, Bartlett T, Oates PJ, Millward BA, Demaine AG 2003 The response of antioxidant genes to hyperglycemia is abnormal in patients with type 1 diabetes and diabetic nephropathy. Diabetes 52:846–851
29. Kim-Schulze S, McGowan KA, Hubchak SC, Cid MC, Martin MB, Kleinman HK, Greene GL, Schnaper HW 1996 Expression of an estrogen receptor by human coronary artery and umbilical vein endothelial cells. Circulation 94:1402–1407
30. Elhadd TA, Khan F, Kirk G, McLaren M, Newton RW, Greene SA, Belch JJ 1998 Influence of puberty on endothelial dysfunction and oxidative stress in young patients with type 1 diabetes. Diabetes Care 21:1990–1996
31. Borras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann GE, Vina J 2005 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade. Aging Cell 4:113–118
32. Silverstein J, Klingensmith G, Copeland K, Plotnick L, Kaufman F, Laffel L, Deeb L, Grey M, Anderson B, Holzmeister LA, Clark N 2005 Care of children and adolescents with type 1 diabetes: a statement of the American Diabetes Association. Diabetes Care 28:186–212
33. Snell-Bergeon JK, Hokanson JE, Jensen L, MacKenzie T, Kinney G, Dabelea D, Eckel RH, Ehrlich J, Garg S, Rewers M 2003 Progression of coronary artery calcification in type 1 diabetes: the importance of glycemic control. Diabetes Care 26:2923–2928
34. Orchard TJ, Olson JC, Erbey JR, Williams K, Forrest KY, Smithline Kinder L, Ellis D, Becker DJ 2003 insulin resistance-related factors, but not glycemia, predict coronary artery disease in type 1 diabetes: 10-year follow-up data from the Pittsburgh Epidemiology of Diabetes Complications Study. Diabetes Care 26:1374–1379
35. Ulker S, McMaster D, McKeown PP, Bayraktutan U 2004 Antioxidant vitamins C and E ameliorate hyperglycaemia-induced oxidative stress in coronary endothelial cells. Diabetes Obes Metab 6:442–451
36. Beckman JA, Goldfine AB, Gordon MB, Creager MA 2001 Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. Circulation 103:1618–1623