Defective Plasma Antioxidant Defenses and Enhanced Susceptibility to Lipid Peroxidation in Uncomplicated IDDM

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Oxidative stress is postulated to be increased in patients with IDDM. Accumulating evidence suggests that oxidative cell injury caused by free radicals contributes to the development of IDDM complications. On the other side, a decreased efficiency of antioxidant defenses (both enzymatic and nonenzymatic) seems to correlate with the severity of pathological tissue changes in IDDM. Thus, we determined plasma antioxidant defenses, measuring the total radical-trapping antioxidant capacity (TRAP) and the two markers of oxidative stress, lipid hydroperoxides (ROOHs) and conjugated dienes, in 72 patients with well-controlled IDDM and without evident complications, compared with 45 nondiabetic subjects. Compared with control subjects, IDDM patients showed significantly reduced plasma TRAP (669 ± 131 vs. 955 ± 104 μmol/l, $P < 0.001$) and significantly increased levels of ROOHs (7.13 ± 2.11 vs. 2.10 ± 0.71 μmol/l, $P < 0.001$) and conjugated dienes (0.0368 ± 0.0027 vs. 0.0328 ± 0.0023 arbitrary units [AU], $P < 0.01$), especially in the *trans-trans* conformation (0.0340 ± 0.0028 vs. 0.0259 ± 0.0022 AU, $P < 0.001$), with a concurrent reduction of conjugated dienes in the *cis-trans* conformation (0.0028 ± 0.0011 vs. 0.0069 ± 0.0012 AU, $P < 0.001$). The oxidative parameters studied did not appear to be correlated with metabolic control (HbA_{1c} levels) and lipid profile (cholesterol or triglyceride levels). The reduced TRAP and the increased ROOH and conjugated diene plasma levels, together with the decreased ratio of *cis-trans/trans-trans* conjugated dienes, which reflects an altered redox status of plasma, indicate that in IDDM patients, oxidative stress is enhanced and antioxidant defenses are defective, regardless of diabetes duration, metabolic control, or presence of complications.
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ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid); AER, albumin excretion rate; AU, arbitrary units; BHT, butylate hydroxytoluene; ROOH, lipid hydroperoxide; ROS, reactive oxygen species; TBA, thiobarbituric acid; TPP, triphenylphosphine; TRAP, total radical-trapping antioxidant capacity.

There is currently great interest in the potential contribution of increased oxidative stress to the development of complications in diabetes (1–4). Increased presence of reactive oxygen species (ROS) has also been implicated in the pathogenesis of type 1 diabetes (5–8). There are different biochemical pathways strictly related with chronic hyperglycemia, such as nonenzymatic glycation, polyol pathway, and glucose autooxidation, which can result at least in vitro in the production of ROS (2,9–16). On the other side, there can be widespread disturbances of antioxidant defense systems, both enzymatic and nonenzymatic, suggesting that a reduced resistance to free radical–induced tissue damage may also occur (2–4,17–25).

For practical reasons, neither the rate of oxidant production nor the steady-state levels of ROS are easily measured directly ex vivo in biological systems. Thus, steady-state levels, or the extent of accumulation of oxidation products in tissues and plasma or the changes in antioxidant status are commonly used as measures of increased oxidative stress (2,3,5,26). In fact, the measurement of parameters of lipid peroxidation, such as lipid peroxides, conjugated dienes, and malonyldialdehyde (MDA), can be considered the cornerstone of the assessment of oxidative damage in vivo (1–3,27).

The aim of our study was to determine the antioxidant status and the susceptibility to oxidative stress in IDDM patients with well-controlled diabetes and without evident complications, measuring total plasma antioxidant capacity (TRAP), a marker of the efficiency of antioxidant defenses (21–25), and two different indexes of lipid peroxidation (conjugated dienes and lipid hydroperoxides [ROOHs]), which are generated at different stages of the peroxidation cascade. We also investigated the correlation between oxidative stress, degree of glycemic control, and diabetes duration.

RESEARCH DESIGN AND METHODS

We studied more than 300 IDDM patients attending our Diabetic Outpatient Clinic of the Catholic University School of Medicine (mean age 38 ± 8 years). The exclusion criteria were the following: the administration of drugs (such as antihypertensive agents, aldose reductase inhibitors, carnitine, or vitamin supplements); pregnancy; and other current illness (such as hepatic, cardiac or renal disease). Hence, we excluded from the study all the patients with diabetic nephropathy (defined as albumin excretion rate [AER] > 200 μg/min), diabetic neuropathy, hypertension (based on the World Health Organization criteria, diastolic > 90 mmHg; systolic > 140 mmHg), cardiac ischemic disease (as judged by pathological changes in the resting electrocardiogram, a previous history of cardiac angina or myocardial infarction), and peripheral vasculopathy (based on clinical examination and Doppler velocimetry). All patients were evaluated according to the cri-

teria of the EURODIAB IDDM Complications Study (28), and all performed AER, which was determined in three overnight timed collections. Microalbuminuria was hence defined as AER between 20 and 200 μg/min, macroalbuminuria as AER >200 μg/min. A formal examination by ophthalmologists with fundus ophthalmoscopy and, if necessary, fluoroangiography. Actually, all patients with a diabetes duration >5 years had fluoroangiography performed at least once. Neuropathy was studied as follows: *1*) sensory neuropathy was checked measuring the vibration perception threshold (VPT) by biothesiometer on the tip of the great toe and the mean of three readings used for analysis; and *2*) autonomic neuropathy was evaluated by testing cardiovascular reflex responses, according to the criteria of Ewing (29). According to the exclusion criteria, only 72 patients were eligible for the study; 7 of these 72 patients had nonproliferative retinopathy. Only three patients were microalbuminuric with urinary protein excretion between 20 and 200 μg/min, without any other clinical complication and not taking any drug. The characteristics of the study subjects are shown in Table 1. The age and sex distributions within the control group, recruited from staff at the Catholic University School of Medicine, were comparable to those of IDDM patients. Cigarette smoking and diet habits as well as physical activity of diabetic patients were comparable to those of control subjects.

Reagents. Ammonium ferrous sulfate, xylenol orange, H_2O_2, butylate hydroxytoluene (BHT), and myoglobin (horse heart) were obtained from Sigma (St. Louis, MO). Triphenylphosphine (TPP) was obtained from Aldrich (Milwaukee, WI), and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was obtained from Fluka (Buchs, Switzerland). All general chemicals and reagents were of the highest purity available.

Clinical laboratory measurements. To assess glycemic control, we measured HbA_{1c} by high-performance liquid chromatography (HPLC). Plasma cholesterol, triglycerides, and uric acid were measured by standard techniques.

Preparation of plasma. Venous blood was collected after a 12-h overnight fast into sampling vials containing heparin for preparation of plasma. Plasma samples were stored under nitrogen at −70°C and used within 1 month for analysis of TRAP and measurement of conjugated dienes and ROOHs. Freezing of the plasma did not affect the results of the assays performed.

Measurements of plasma antioxidant capacity (TRAP). The assay, as originally described by Rice-Evans and Miller (30), is based on the quenching of the ABTS radical cation ($ABTS^{\bullet+}$) by antioxidants. In the method, $ABTS^{\bullet+}$ is produced by the interaction of ABTS with ferrylmyoglobin radical species, generated by the activation of metmyoglobin with H_2O_2. In our assay procedure, ABTS (150 μmol/l), metmyoglobin (2.5 μmol/l), and plasma (25 μl) were mixed together, and the reaction was started by the addition of H_2O_2 (75 μmol/l). $ABTS^{\bullet+}$ formation was continuously monitored by absorbance increase at 734 nm, at 20°C. The delay or inhibition-time between the addition of H_2O_2 (time zero) and the onset of absorbance increase ($ABTS^{\bullet+}$ formation) was measured. All the reagents were dissolved in phosphate buffer treated with Cheelex-100 and containing DTPA 0.1 mmol/l to prevent any metal-catalyzed oxidation. The assay was standardized using Trolox, a water-soluble vitamin E analog. Intra- and interassay coefficients of variation for this method were 7.5 and 9.1%, respectively.

Measurements of lipid peroxidation products. Plasma lipids were extracted by a modification of the method of Folch (31), and 3.8 ml of 2:1 (vol/vol) chloroform-methanol mixture was added to 0.2 ml of plasma. The mixture was vigorously mixed (with a vortex) for 2 min and then 1.0 ml of distilled water, acidified to pH 2.5 with 0.1 N HCl, was added. After agitation with a vortex for 2 min, the suspension was centrifuged at 3,000 rpm for 5 min at 4°C. The lower chloroform lipid layer was removed, dried under vacuum in a Savant RC 100 Speed-Vac concentrator (Savant Instruments, Farmingdale, NY), and resuspended in 1.0 ml HPLC–grade methanol for conjugated diene assay or 100 μl HPLC–grade methanol for hydroperoxide measurement.

Hydroperoxides. The hydroperoxide content of plasma was determined with the FOX Version II assay for lipid ROOHs (FOX2) (32,33). This technique relies on the rapid hydroperoxide-mediated oxidation of Fe^2 under acidic conditions. Fe^3 forms a chromofore with xylenol orange, which absorbs strongly at 560 nm. FOX2 reagent was prepared by dissolving xylenol orange and ammonium ferrous sulfate in 250 mmol/l H_2SO_4 to final concentrations of 1 and 2.5 mmol/l, respectively. One volume of this concentrated reagent was added to 9 vol of HPLC–grade methanol containing 4.4 mmol/l BHT to make the working reagent, which comprised 250 μmol/l ammonium ferrous sulfate, 100 μmol/l xylenol orange, 25 mmol/l H_2SO_4, and 4 mmol/l BHT in 90% (vol/vol) methanol. The working reagent was routinely calibrated against solution of H_2O_2 of known concentration.

Aliquots (90 μl) of plasma lipid extract in HPLC–grade methanol were transferred into 1.5-ml microcentrifuge vials. TPP in methanol (10 μl of 10 mmol/l) was added to the blank samples to selectively reduce ROOHs to hydroxy derivatives having no reactivity with Fe^2. Methanol (10 μl) was added to the test sample. All vials were then vortex-mixed and incubated at room temperature for 30 min before the addition and mixing of 900 μl FOX2 reagent. After incubation at room temperature for a further 30 min, the vials were centrifuged at 12,000*g* for 10 min. The absorbance of the supernatant was then read at 560 nm. Hydroperoxide content in the plasma samples was determined as a function of the mean absorbance difference of samples with and without elimination of ROOHs by TPP. Intra- and interassay coefficients of variation for this method were 5.0 and 7.5%, respectively.

Conjugated dienes. Conjugated dienes were measured by second derivative spectrophotometry (34–36), which allows great sensitivity, since the small peak attributable to conjugated dienes on the tail of absorption caused by total lipid at 230- to 235-nm wavelength translates into sharp minima, which are more easily measurable. Furthermore, this technique permits a discrimination between the different configurations of conjugated dienes (e.g., *cis-trans* and *trans-trans*), giving supplementary information on the distribution of conjugated diene isomers present in the sample (37–40). Absorbance spectra of plasma lipid extract in HPLC–grade methanol were read against a methanol blank with an HP 8452A diode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA), and the second derivative spectra were obtained. Minima at 232 and 246 nm were ascribed to the *trans-trans* and *cis-trans* conjugated dienes isomers, respectively (34,36,37). These minima were quantified in arbitrary units as $d^2A/d\lambda^2$, which represents the measurement from minima to adjacent maxima at the higher wavelength (34). The ratio of conjugated diene measurements at 246- and 232-nm minima of the derivative spectra, which can provide an indirect evaluation of plasma antioxidant capacity (34,37), was then calculated. Intra- and interassay coefficients of variation for this method were 7.5 and 10.2%, respectively.

Statistical analysis. Statistical calculations were performed using StatView software package (version 4.01) (Abacus Concepts, Berkeley, CA). Values are given as means ± SD. Comparisons between diabetic and control groups were made using the Student's *t* test. Linear regression analysis was used for detecting relationships between TRAP values, conjugated dienes, and lipid hydroperoxides levels.

RESULTS

Our data showed that IDDM patients had significantly lower levels of total plasma antioxidant defenses and increased levels of lipid peroxidation products, compared with control subjects. As shown in Table 2, TRAP values were 669 ± 131 vs. 955 ± 104 μmol/l ($P < 0.001$), and lipid hydroperoxides were 7.13 ± 2.11 vs. 2.10 ± 0.71 μmol/l ($P < 0.001$). Total conjugated dienes were increased (0.0368 ± 0.0027 vs. 0.0328 ± 0.0023 arbitrary units [AU], $P < 0.01$), especially in the *trans-trans* conformation (0.0340 ± 0.0028 vs. 0.0259 ± 0.0022 AU, $P < 0.001$) with a concurrent reduction of conjugated dienes in the *cis-trans* conformation (0.0028 ± 0.0011 vs. 0.0069 ± 0.0012 AU, $P < 0.001$). This characteristic pattern of conjugated dienes is clearly shown in Fig. 1.

Plasma TRAP of IDDM patients showed a significant inverse correlation with ROOHs ($r^2 = 0.755$, $P < 0.0001$) (Fig. 2*A*) and a relevant direct correlation with the 246-nm peak height of *cis-trans* conjugated diene isomers ($r^2 = 0.418$, $P < 0.0001$) (Fig. 2*B*), confirming the strict correlation between decreased antioxidant defenses and increased oxidative stress. Moreover, as shown in Fig. 2*C*, there was also a significant relationship between hydroperoxide levels and the 246-nm peak height of conjugated dienes in IDDM patients ($r^2 = 0.351$, $P < 0.0001$).

The ratio of peak heights at 246 nm (*cis-trans* isomers) over 232 nm (*trans-trans* isomers) of IDDM patients showed a significant difference, compared with that of control subjects (0.081 ± 0.021 vs. 0.271 ± 0.042, $P < 0.0001$).

As shown in Table 1, there were no significant differences between normal and diabetic subjects in the levels of plasma triglycerides (0.91 ± 0.26 vs. 0.86 ± 0.21 mmol/l, NS), total cholesterol (4.79 ± 0.74 vs. 4.84 ± 0.91 mmol/l, NS), HDL cholesterol (1.56 ± 0.41 vs. 1.61 ± 0.39 mmol/l, NS), LDL cholesterol (2.74 ± 0.18 vs. 2.77 ± 0.40 mmol/l, NS), and uric acid (0.18 ± 0.06 vs. 0.20 ± 0.05 mmol/l, NS), one of the principal constituents of the total antioxidant pool. Furthermore, there was no correlation between these parameters and the markers of

TABLE 1
Clinical characteristics of control and IDDM subjects

	Control	IDDM	*P* value
n	45	72	
Sex (M/F)	27/18	41/31	NS
Age (years)	32 ± 7	34 ± 9	NS
Duration (years)	—	14 ± 8	—
HbA_{1c} (4.5–6.5%)	—	7.5 ± 1.2	—
BMI (kg/m^2)	23.1 ± 2.2	23.3 ± 2.5	NS
Triglycerides (0.2–2.0 mmol/l)	0.91 ± 0.26	0.86 ± 0.21	NS
Cholesterol (3.36–5.18 mmol/l)	4.79 ± 0.74	4.84 ± 0.91	NS
HDL cholesterol (>1.16 mmol/l)	1.56 ± 0.41	1.61 ± 0.39	NS
LDL cholesterol (<3.36 mmol/l)	2.74 ± 0.18	2.77 ± 0.40	NS
Uric acid (0.14–0.41 mmol/l)	0.18 ± 0.06	0.20 ± 0.05	NS
Systolic blood pressure (mmHg)	115 ± 11	118 ± 10	NS
Diastolic blood pressure (mmHg)	72 ± 11	75 ± 10	NS

Data are means ± SD. IDDM patients were all treated with intensified insulin therapy (three or four daily injections).

oxidative stress, such as conjugated dienes, ROOHs, and TRAP.

The mean values of HbA_{1c} were 7.5 ± 1.2% in IDDM patients. No correlation was found between metabolic control and the levels of the markers of oxidative stress, such as conjugated dienes, ROOHs, and TRAP, even if the patients were divided into two groups according to their HbA_{1c} values (≤7.5% and >7.5%). Furthermore, these parameters of oxidative stress did not correlate with age, sex, and diabetes duration.

DISCUSSION

In this paper, we investigated plasma antioxidant status and the susceptibility to lipid peroxidation in IDDM patients with good metabolic control, without chronic diabetic complications, and drug assumption, to determine if the alterations in the antioxidant defenses and the increase in lipoperoxidation products described in IDDM patients were related to glycemic control or diabetes duration.

Compared with control subjects, IDDM patients had significantly lower TRAP values (Table 2), meaning that their antioxidant defenses were significantly decreased. In fact, TRAP is largely determined by the plasma content of water-soluble antioxidants like uric acid, accounting for >60% of TRAP, ascorbic acid (3.5%), protein thiols (15%), bilirubin and lipid-soluble antioxidants like α-tocopherol (5%), β-carotene, and ubiquinol and can reflect the real plasma chain-breaking antioxidants status (21,22,30,41). There were no significant differences between the two groups of subjects in plasma levels of uric acid, one of the principal constituents of the total antioxidant pool (Table 1). This was not surprising since antioxidants could act cooperatively in vivo, in a process that involves antioxidant regeneration (1,4,42), so as to provide greater protection to the organism against radical damage than could be afforded by any single antioxidant acting alone (41,43). In this regard, we found no correlation between plasma uric acid and markers of oxidative stress, such as conjugated dienes, ROOHs, and TRAP in IDDM patients.

There have been previous studies evaluating plasma antioxidants in diabetic patients, (17–23). However, most studies focused on a limited number of individual antioxidants (24) and, in some studies, the type of diabetes was not specified (20). Other studies compared lipid peroxidation products in normal plasma and tissues with those from diabetic individuals without specifying whether complications were present (18,44). Several others used the nonspecific thiobarbituric acid (TBA) assay or the measurement of conjugated dienes by simple absorption spectroscopy; such studies have been criticized for their ambiguity (44–51), and their results were conflicting (20,45,47). Second derivative spectrophotometry (34–36) allows great sensitivity and permits a discrimination between the different configurations of conjugated diene isomers (i.e., *cis-trans* and *trans-trans*) present in the sample (37–40). In fact, compared with control subjects, IDDM patients showed increased levels of overall conjugated dienes, especially in the *trans-trans* conformation with a

TABLE 2
Plasma antioxidant activity (TRAP), lipid hydroperoxides, and conjugated dienes levels in IDDM patients and control subjects

	Control	IDDM	*P* value
TRAP (μmol/l)	955 ± 104	669 ± 131	<0.001
Hydroperoxides (μmol/l)	2.10 ± 0.71	7.13 ± 2.11	<0.001
Conjugated dienes (AU) $d^2A/d\lambda^2$			
232 nm (*trans-trans* isomers)	0.0259 ± 0.0022	0.0340 ± 0.0028	<0.001
246 nm (*cis-trans* isomers)	0.0069 ± 0.0012	0.0028 ± 0.0011	<0.001
232 nm + 246 nm	0.0328 ± 0.0023	0.0368 ± 0.0027	<0.01

Data are means ± SD. Conjugated dienes are expressed as A.U. ($d^2A/d\lambda^2$) obtained from the second derivative ultraviolet spectra.

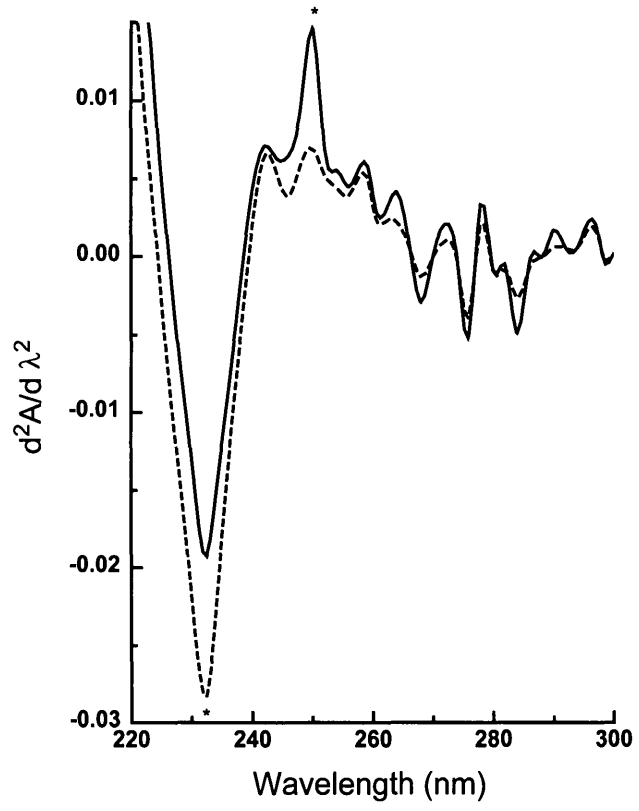

FIG.1. Second derivative of ultraviolet spectrum of conjugated dienes extracted from plasma of IDDM patients (– – –) and control subjects (——). The *trans-trans* and *cis-trans* isomers are revealed by two sharp and distinct signals, with their distinctive absorption minima at 232 and 246 nm, respectively. *P < 0.001.

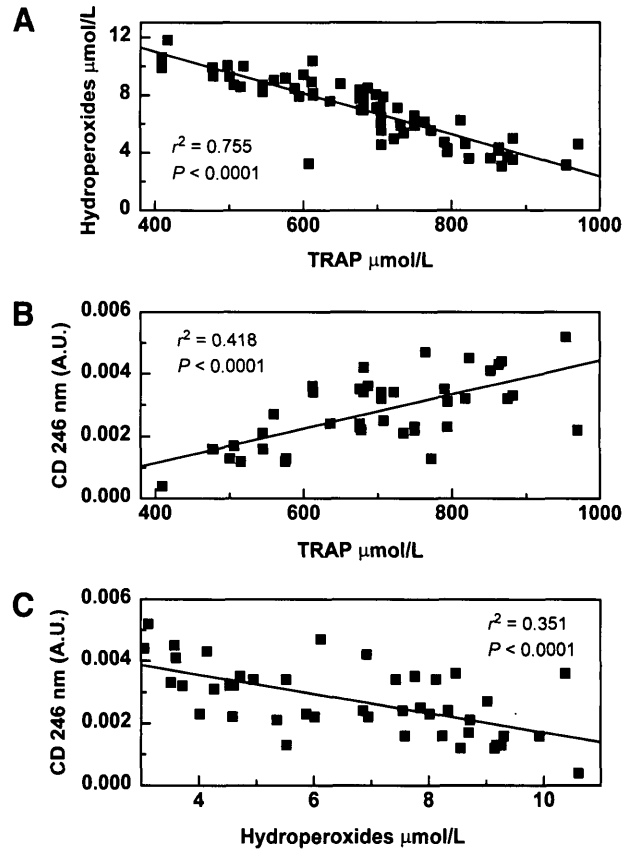

FIG.2. Correlation between antioxidant capacity (TRAP) values and hydroperoxides levels (*A*) and 246 nm peak height of conjugated dienes (*B*), expressed as arbitrary units ($d^2A/d\lambda^2$); correlation (*C*) between hydroperoxides levels and 246-nm peak height of conjugated dienes, expressed as arbitrary units ($d^2A/d\lambda^2$) in IDDM patients.

concurrent reduction of conjugated diene in the *cis-trans* conformation (Table 2). As shown in Fig. 1, plasma of control subjects has presumably a relatively high concentration of hydrogen donors: the oxidative reaction is directed toward the formation of *cis-trans* products and the minimum at 246 nm is clearly evident together with the minimum at 232 nm of dietary conjugated dienes. In diabetic patients, the hydrogen-donating ability of plasma decreases because of consumption of the antioxidant donors and the kinetic equilibrium of the oxidation is shifted toward *trans-trans* products, with the increase of the minimum at 232 nm and the concomitant reduction of the minimum at 246 nm. Moreover, a large depletion of antioxidants is confirmed by the decrease in the ratio of the minima at 246 nm (*cis-trans* isomers) over 232 nm (*trans-trans* isomers), which can provide an indirect evaluation of plasma antioxidant capacity (34,37).

Plasma lipid hydroperoxides were also increased in IDDM patients compared with control subjects (Table 2), giving strength to the concept of an increased lipid peroxidation: in fact, ROOHs are formed following the rearrangement of double bonds and formation of conjugated dienes, on oxygen incorporation and further propagation reactions. Furthermore, as there were no significant differences in the plasma levels of triglycerides between normal and diabetic subjects (see Table 1), the reported rise in plasma ROOHs and conjugated dienes was not due to an increase in the content of this class of circulating lipids. The tight correlation between decreased antioxidant defenses and increased lipid peroxidation in IDDM patients (1,2,4,42,52) was supported by the significant inverse correlation of plasma TRAP with ROOHs (Fig. 2*A*), by a relevant direct correlation between values of TRAP and 246-nm peak height of *cis-trans* conjugated dienes isomers (Fig. 2*B*) and by a significant inverse correlation between ROOH levels and 246-nm peak height of conjugated dienes (Fig. 2*C*).

Recently, Jenkins et al. (25) reported a normal susceptibility of LDL to in vitro oxidation in well-controlled IDDM patients. Their data are only apparently in contrast with ours, since we measured in vivo conjugated dienes and lipid hydroperoxides, which are indexes of a more general lipoperoxidative process involving all the classes of lipoproteins. Furthermore, their study focused on a limited number of subjects. In contrast, Tsai et al. (22) reported reduced TRAP values and increased susceptibility of LDL to in vitro oxidation in poorly controlled IDDM patients, suggesting that hyperglycemia would render LDL more susceptible to in vitro oxidation. The fact that we found no correlation between glycemic control, as expressed by HbA_{1c} levels, diabetes duration, and markers of oxidative stress, such as conjugated dienes, ROOHs, and TRAP, can be explained considering that factors other than simple hyperglycemia, expressed by HbA_{1c} (i.e., genetic susceptibility), can contribute to oxidative stress. It can be postulated that in diabetes, a continuing cycle of metabolic stress, tissue damage, and cell death would lead to increased free radical production and compromised total plasma antioxidant capacity, which would further enhance

oxidative stress and finally determine the onset of complications (1–8,53). In this view, the different susceptibility of diabetic patients to complications could be a function of the endogenous antioxidant status; thus ROOHs, conjugated dienes, and TRAP measurements can be considered as early markers of oxidative damage, which can be useful to monitor the evolution of the disease. Further longitudinal studies, with assessment of oxidative damage at several time points are hence necessary to reach a better understanding of the development of diabetic complications and the management of their various and complex manifestations.

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