Oxidative stress and interleukin-6 secretion during the progression of type 1 diabetes

Estresse oxidativo e secreção de interleucina-6 durante a progressão do diabetes tipo 1

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ABSTRACT

Objective: To evaluate inflammatory, oxidizing, and reducing responses during the progression of type 1 *diabetes mellitus* (T1DM) in patients without chronic complications. **Subjects and methods:** Plasma antioxidant status, reactive oxygen species (ROS), and interleukin-6 (IL-6) were measured in 42 patients with T1DM and in 24 healthy subjects. **Results:** Significant increases were detected in the median values of ROS and IL-6 in patients with T1DM compared with healthy subjects (ROS ~ 4,836 *vs.* 2,036 RLU/min, respectively; P < .05: IL-6 ~ 14.2 *vs.* 9.7 pg/mL, respectively; P = .002). No significant between-group differences (P > 0.05) were observed in oxidizing responses or in IL-6 concentrations when diabetic patients were grouped according to time after diagnosis (0 - 10, 10 - 20 and > 20 years). Plasma antioxidant responses were similar in patients with T1DM and in healthy subjects. **Conclusions:** Our results demonstrate that oxidizing and inflammatory responses are increased at the onset of T1DM, but remain unchanged during disease progression. These findings suggest that functional changes involved in diabetic complications may commence in the first years after diagnosis. Arq Bras Endocrinol Metab. 2012;56(7):441-8

Keywords

Type 1 diabetes; disease progression; oxidative stress; plasma antioxidant status

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RESUMO

Objetivo: Avaliar as respostas inflamatória, oxidativa e redutora na progressão do diabetes melito tipo 1 (DM1) em pacientes sem complicações crônicas. **Sujeitos e métodos:** Capacidade antioxidante do plasma, espécies reativas de oxigênio (ROS) e interleucina-6 (IL-6) foram avaliadas em 42 pacientes com DM1 e 24 indivíduos saudáveis. **Resultados:** Aumentos significativos foram detectados nas medianas de ROS e IL-6 em pacientes com DM1 comparados com indivíduos saudáveis (ROS ~ 4.836 *vs.* 2.036 RLU/min, respectivamente, P < 0,05: IL-6 ~ 14,2 *vs.* 9,7 pg/mL, respectivamente, P = 0,002). Diferenças não significativas (P > 0,05) foram observadas na resposta oxidante e IL-6 quando os diabéticos foram agrupados de acordo com o tempo após o diagnóstico (0-10, 10-20 e > 20 anos). A resposta antioxidante do plasma foi semelhante em pacientes com DM1 e em indivíduos saudáveis. **Conclusões:** Nossos resultados demonstram que as respostas oxidante e inflamatória estão aumentadas desde o início do DM1, mas mantêm-se inalteradas durante a progressão da doença, sugerindo que as mudanças funcionais envolvidas nas complicações diabéticas podem começar nos primeiros anos após o diagnóstico. Arq Bras Endocrinol Metab. 2012;56(7):441-8

Descritores

Diabetes tipo 1; progressão da doença; estresse oxidativo; capacidade oxidante do plasma

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INTRODUCTION

Type 1 *diabetes mellitus* (T1DM) is an inflammatory disease of the pancreatic islets in which the destruction of beta cells is mediated by cytotoxic T cells, self antibodies, and inflammatory mediators (1). High levels of inflammatory biomarkers can be detected in patients that have been recently diagnosed with T1DM, and this indicates that the inflammatory response is activated during very early stages of the disease (2,3).

It has been suggested that a number of different hyperglycemia-induced biochemical mechanisms may be involved in the pathogenesis of complications that are characteristic of diabetes. In this context, it is known that hyperglycemia is linked with the activation of protein kinase C (PKC) and nuclear factor kappa B, enhanced polyol activity, increased formation of advanced glycation end-products (AGEs), and elevated flux through the hexosamine pathway (4,5). A mechanism that is common to all the pathways implicated in these oxidative processes involves the generation of reactive oxygen species (ROS) (6), which lead to changes in blood flow, vascular permeability, and angiogenesis. Increased levels of ROS stimulate PKC via diacylglycerol (DAG), and give rise to greater production of inflammatory cytokines, mainly interleukin-6 (IL-6), which is considered the primary mediator of acute inflammatory response. However, all inflammatory cytokines exhibit cytotoxic and cytostatic activities and have the ability to induce apoptosis in pancreatic islet cells (7). It has been hypothesized that alterations in oxidative metabolism without simultaneous increases in antioxidant response are characteristic of oxidative stress which, in endothelial cells, results in endothelial dysfunction and vascular damage (4,6).

Over the last few decades, studies on atherosclerosis and other inflammatory disorders have shown that levels of inflammatory markers can be used to predict cardiovascular risk, thus reinforcing the need to define the impact of inflammatory activity in diabetes (8). While the main determinants of tissue injury in diabetes are believed to be the duration and severity of hyperglycemia (9), there are very few reports on the changes that occur in inflammatory, oxidizing, and reducing responses during the progression of T1DM. Several authors have stated that, in patients with T1DM, no significant pathological consequences of diabetes can be detected earlier than 5 years from onset (10), even though significant increases in ROS generation can be detected in patients at the onset of the disease (11). These findings suggest that the structural and functional changes involved in diabetic complications may commence at the onset of the hyperglycemic trigger, and it is possible that, in the presence of persistent acute peaks or chronic hyperglycemia, inflammatory metabolic and immunological pathways can become activated at all times after diagnosis (12).

In the light of the information presented above, the objective of the present study was to investigate inflammatory and antioxidant status in patients with T1DM but without chronic complications, during the first years after diagnosis (0-10 years) and during further progression of the disease (10-20 and > 20 years after diagnosis).

METHODS

Details of the study were submitted to, and approved by the Ethical Committee of the Santa Casa Hospital in Belo Horizonte, Minas Gerais, Brazil. The investigation was conducted according to the principles of the Declaration of Helsinki, and written informed consent was obtained from each participant before the study.

Subjects

The study population was made of 42 patients with T1DM and 24 healthy subjects. Diabetic patients exhibiting micro- or macrovascular complications or ketoacidosis in the previous year, and those who were taking statin and/or metformin and/or vitamins, or suffering from infection, dementia, inflammation, or malignant disease, or addicted to smoking or alcohol, or were pregnant, were excluded from the study. Patients with T1DM who were selected to be included in the study showed clinical onset of the disease 13.03 ± 9.42 years before, and presented history of diabetic ketoacidosis and/or were positive for glutamic acid dehydrogenase (GAD) antibodies and C peptide (< 0.5 ng/mL). The patients selected for the study, all of whom were receiving intensive insulin therapy by means of multiple daily injections, were divided into three groups according to the time elapsed since diagnosis, namely, 0-10 years, 10-20 years, and > 20 years. Clinical and biochemical evaluations were performed in all participants according to standard procedures.

Preparation of plasma and granulocyte samples

Blood samples were collected between 7 and 8 am using a standard venipuncture. Plasma was obtained by centrifugation (200 g, 15 min, at room temperature) of

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heparinized venous blood (10.0 mL), and was stored at –20°C until the moment of analysis. Granulocytes were purified using a Ficoll-Hypaque gradient according to the method described by Bicalho and cols. (13).

Estimation of ROS production in granulocytes

Luminometric determination of the modulation of granulocyte ROS generation was carried out using a quantitative chemiluminescence assay. Granulocytes (1 x 10^6 cells/mL; 200 µL) suspended in phosphate buffered saline (PBS; 300 µL) were transferred to an unsealed luminescence tube together with luminol (200 µL) dissolved in 0.4 M dimethyl sulfoxide. Chemiluminescence of the assay mixture was monitored for 30 min. After that, ROS production was stimulated by the addition of a 10-µL aliquot of 10^{-4} M phorbol 12,13-dibutyrate (PDB), and chemiluminescence monitored for other 30 min (13). Results of the assay were expressed in relative light units (RLU) per minute.

Evaluation of plasma antioxidant status

Plasma antioxidant status was determined from the direct reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5--diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) according to the method of Medina and cols. (14). Briefly, a 200-µL aliquot of plasma was mixed with 25 µL of MTT solution (5.0 mg/mL in PBS), the final volume was adjusted to 500 µL with PBS, and the whole mixture was incubated for 120 min at 37°C. The reaction was subsequently terminated by the addition of 1.0 mL of 0.04 M hydrochloric acid in isopropanol. The mixture was centrifuged (200 g, 10 min) and absorbance of the supernatant measured at 570 nm.

Determination of IL-6

Plasma concentrations of IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) using Assay Designs (Enzo Life Sciences) Human IL-6 Enzyme Immunometric kits.

Statistical analyses

The Shapiro-Wilk test was applied in order to verify that data were normally distributed and, where applicable, mean values were compared using Student's t-tests for paired samples. Between-group comparisons of median values of inflammatory biomarkers and plasma reducing responses of diabetic patients were performed using the non-parametric Mann-Whitney test. Within--group correlations were determined using Spearman rank correlation tests. In all cases, statistical significance was accepted at P values < 0.05.

RESULTS

Baseline characteristics of the study populations

Clinical characteristics of patients with T1DM and healthy subjects were very similar, as shown in Table 1. In relation to biochemical characteristics, fasting plasma glucose values were significantly increased in diabetic patients in comparison with healthy subjects ($P < .05$), while levels of glycated hemoglobin (HbA1c) in patients with T1DM (8.84%) were somewhat higher than the accepted range for healthy subjects (< 7%). When clinical and biochemical characteristics of the population of diabetic patients were distributed according to the time elapsed since diagnosis (Table 2), significant between--group differences were observed in some parameters, although these were not significant in clinical practice.

Inflammatory biomarkers and oxidative stress

A significant difference was detected between patients with T1MD and healthy subjects in relation to the median values of ROS production in unstimulated granulocytes (4,836 *versus* 2,036 RLU/min, respectively; $P < 0.05$) (Figure 1A). The presence of phorbol ester stimulated granulocyte ROS production differently in diabetic patients and healthy subjects (22369 and 5780 RLU/min, respectively; $P < 0.05$) (Figure 1B). Additionally, IL-6 plasma levels of patients with T1MD were significantly higher than those of healthy subjects (14.2 *versus* 9.7 pg/mL, respectively; $P = 0.002$) (Figure 1C). In contrast, no significant difference ($P > 0.05$) was observed between diabetic patients and healthy subjects in relation to plasma reducing responses (as determined by direct reduction of MTT), although patients with T1DM showed a slightly lower response (median values 0.3 *versus* 0.29 OD_{570}) (Figure 1D).

When the levels of inflammatory biomarkers and plasma reducing responses determined in patients with T1DM were distributed according to time elapsed since diagnosis, no significant between-group differences ($P > 0.05$) were detected (Figures 2A-C). However, values of the inflammatory markers of the three groups of time elapsed since diagnosis, when considered individually, were significantly different from the correspon-

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ding levels measured in healthy subjects. In contrast, plasma antioxidant status did not differ significantly ($P > 0.05$) between healthy subjects and the three individual groups of patients with T1DM.

Table 1. Clinical and biochemical characteristics of the studied population

Parameters	Patients with T1DM (n = 42)	Healthy patients (n = 24)	P values
Disease duration (years)	13.03 ± 9.42	NA	NA
Female/male ratio	22/20	12/12	-
Age (years)	27.7 ± 9.4	32.7 ± 10.6	ns
Body mass index (kg/m²)	22.9 ± 3.6	23.1 ± 4.6	ns
Waist circumference (cm)	77.9 ± 9.8	78.5 ± 9.1	ns
Waist-hip ratio	0.84 ± 0.08	0.82 ± 0.08	ns
Systolic pressure (mmHg)	117 ± 16	119.2 ± 13.5	ns
Diastolic pressure (mmHg)	74 ± 12	79.79 ± 8.6	ns
Fasting glucose (mg/dL)	179 ± 96	87.41 ± 7.6	< .05
Total cholesterol (mg/dL)	173.9 ± 39.3	177.16 ± 33.4	ns
High density lipoprotein (mg/dL)	52.8 ± 14.2	49.2 ± 11.5	ns
Low density lipoprotein (mg/dL)	104.2 ± 29.6	108.6 ± 29.1	ns
Very low density lipoprotein (mg/dL)	16.9 ± 11.7	21.12 ± 11.2	ns
Triglycerides (mg/dL)	85.7 ± 53.3	102.7 ± 50.7	ns
Glycated hemoglobin (%)	8.84 ± 2.95	NA	NA
Uric acid (mg/dL)	4.2 ± 1.9	4.6 ± 1.2	ns
Albumin (g/dL)	4.4 ± 0.6	4.4 ± 0.6	ns
Insulin dose (IU/kg/day)	0.8 ± 0.3	NA	NA

NA: not applicable; ns: not significant.
Data are expressed as means ± standard deviation. Significant differences between the groups were determined using Student's *t* test ($P < 0.05$).

Table 2. Clinical and biochemical characteristics of patients with T1DM stratified according to time elapsed after diagnosis

Parameters	< 10 years (n = 18)	10 - 20 years (n = 13)	> 20 years (n = 11)
Disease duration (years)	4.3 ± 2.2*	15.2 ± 2.8**	24.7 ± 7.2‡
Female/male ratio	9/9	8/5	5/6
Age (years)	21.4 ± 6.7*	26.5 ± 4.7†	39.6 ± 5.7‡
Body mass index (kg/m²)	21.8 ± 3.5	22.5 ± 2.5	25.1 ± 4.4
Waist circumference (cm)	74.9 ± 7.3	76.8 ± 5.4	84.5 ± 14.3
Waist-hip ratio	0.8 ± 0.1	0.8 ± 0.1†	0.9 ± 0.1‡
Systolic pressure (mmHg)	108.8 ± 9.6*	119.8 ± 15.5	128.2 ± 18.3‡
Diastolic pressure (mmHg)	69.8 ± 8.5*	79.5 ± 13.4	77.7 ± 13.7‡
Fasting glucose (mg/dL)	174.9 ± 101.5	182.7 ± 89.5	181.8 ± 104.9
Total cholesterol (mg/dL)	168.5 ± 37.2	186 ± 49.1	168.5 ± 28.8
High density lipoprotein (mg/dL)	53.4 ± 12.5	55.7 ± 15.5	48.5 ± 27.3
Low density lipoprotein (mg/dL)	101.6 ± 30	113.8 ± 30.7	97.1 ± 27.3
Very low density lipoprotein (mg/dL)	13.5 ± 6.5	16.5 ± 14.8	22.9 ± 12.8
Triglycerides (mg/dL)	67.3 ± 32.3	70.5 ± 38.1†	133.9 ± 68.2‡
Glycated hemoglobin (%)	9.7 ± 3.5	7.9 ± 2.7	8.31 ± 1.7
Uric acid (mg/dL)	3.3 ± 1.1	3.7 ± 1.4†	6.1 ± 2.2‡
Albumin (g/dL)	4.6 ± 0.4	4.2 ± 0.8	4.3 ± 0.6
Insulin dose (IU/kg/day)	1.0 ± 0.3*	0.7 ± 0.2	0.6 ± 0.2‡

Data are expressed as means ± standard deviation. Significant differences between the groups (* < 10 and 10 – 20 years; † 10 – 20 and > 20 years; ‡ < 10 and > 20 years) were determined using Student's *t* test ($P < 0.05$).

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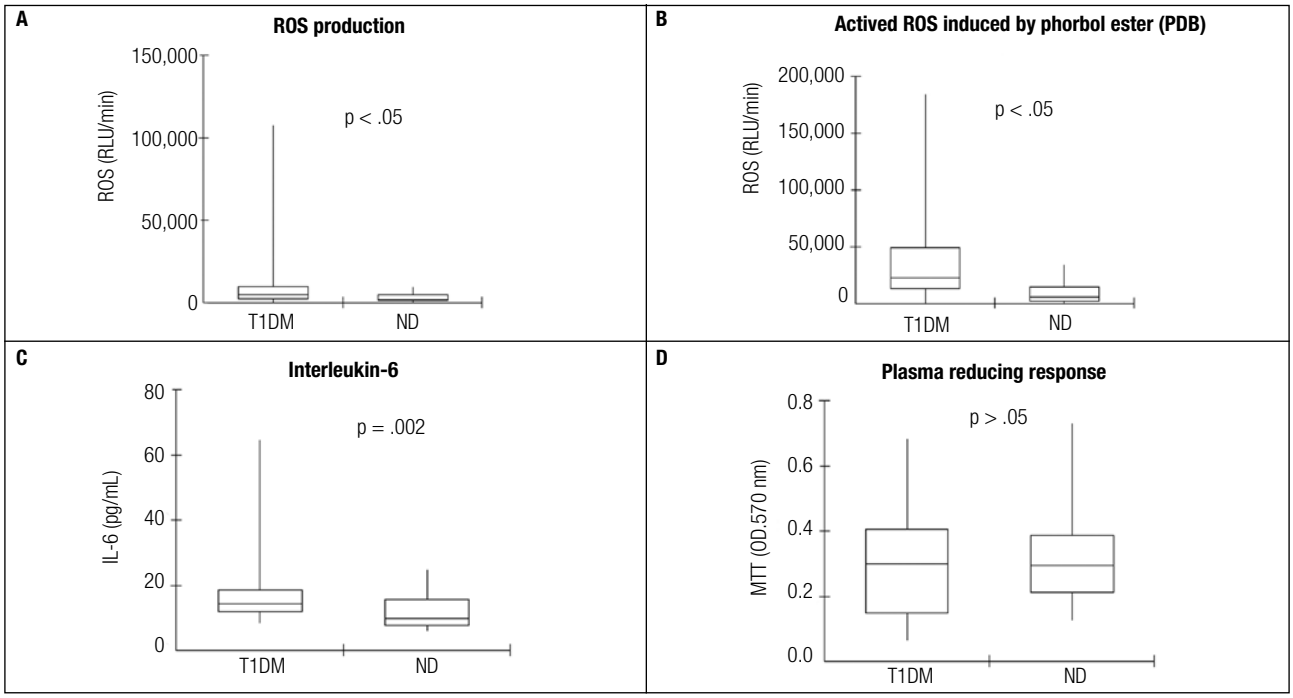

Figure 1. Inflammatory biomarkers and plasma reducing responses in patients with T1DM and in healthy subjects, A: production of reactive oxygen species (ROS) in the absence of phorbol 12,13-dibutyrate (PDB); B: production of ROS in the presence of 10^{-4} M PDB; C: plasma concentration of interleukin-6 (IL-6); D: reducing response evaluated by direct reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, ND: Non diabetic control; T1DM: Patients with type 1 *diabetes mellitus*; OD: optical density, Statistical analyses were performed using Mann-Whitney test at 5% significance level,

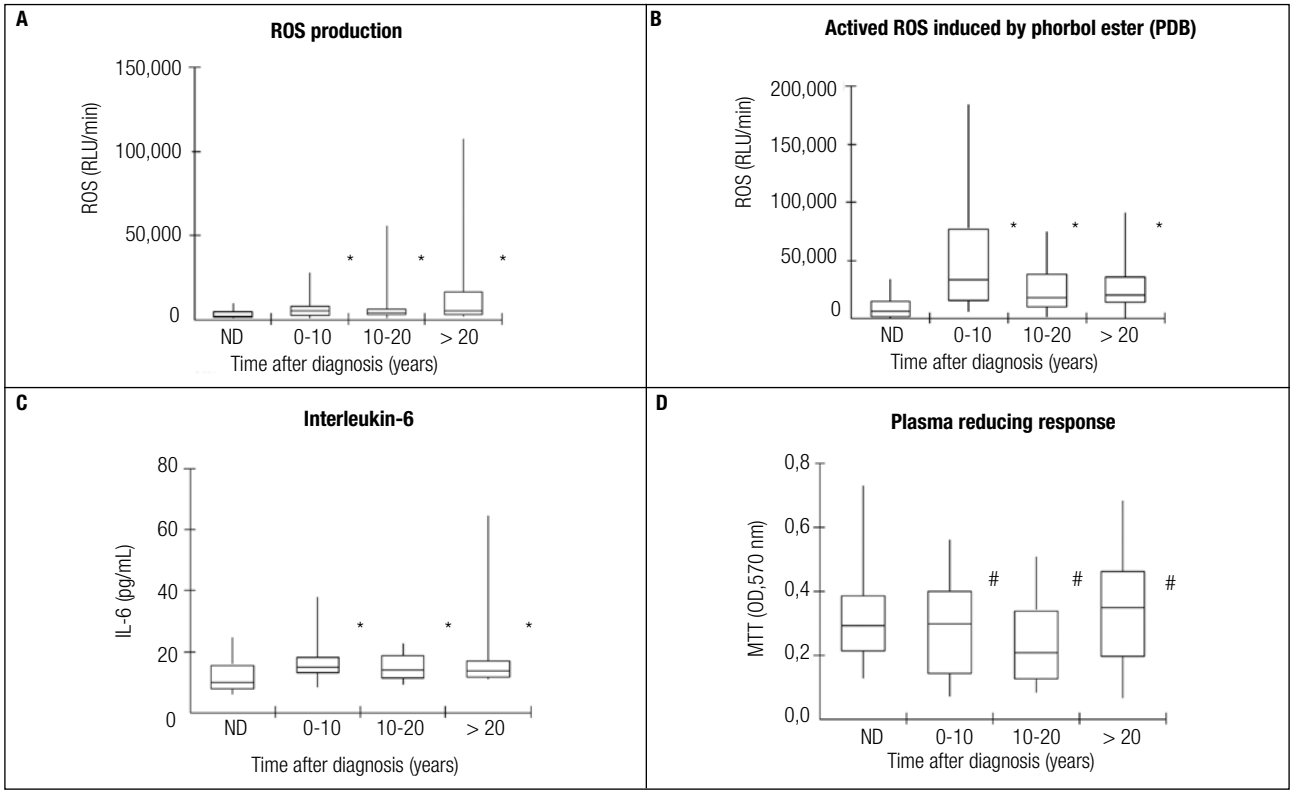

Figure 2. Inflammatory biomarkers and plasma reducing responses in patients with T1DM stratified according to time elapsed after diagnosis and in healthy subjects. A: production of reactive oxygen species (ROS) in the absence of phorbol 12,13-dibutyrate (PDB); B: production of ROS in the presence of 10^{-4} M PDB; C: plasma concentration of interleukin-6 (IL-6); D: reducing response evaluated by direct reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye. ND: non diabetic control; T1DM: patients with type 1 *diabetes mellitus*; OD: optical density. Statistical analyses were performed using Mann-Whitney test at 5% significance level; * P < .05 compared with healthy individuals. # P > .05 compared with healthy individuals.

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Correlations of parameters within the groups of diabetic patients

There were no significant correlations between the levels of inflammatory biomarkers in the diabetic patients, either considered as a single group or when divided according to time since diagnosis, or between these markers and the clinical and biochemical parameters evaluated. Similar results were observed in relation to plasma antioxidant status. Additionally, there were no correlations between fasting glucose, HbA1c and inflammatory biomarkers or plasma antioxidant status.

DISCUSSION

Results obtained in the present study suggest the presence of systemic inflammation in diabetic patients at all moments after T1DM diagnosis. Such condition results from an increased oxidizing metabolic response in the absence of compensating increase in plasma antioxidant status, and is indicative of oxidative stress at disease onset and in long-established T1DM.

A considerable body of evidence has been accumulated over the last few decades supporting the hypothesis that ROS generation, and the resultant oxidative stress, plays a central role in the pathogenesis of diabetic complications. In this context, it is well-documented that pathways stimulated by hyperglycemia (i.e., glucose oxidation, enhanced polyol pathway activity, and formation of complexes between AGEs and their receptors) bring about cell injury in response to oxidative stress (15). In addition, ROS triggers an inflammatory cascade by means of the production of pro-inflammatory cytokines, such as IL-6, which are involved in the pathogenesis of micro- and macrovascular complications. Indeed, these signaling cytokines are often employed as markers of the inflammatory response (16).

Studies focusing on the extent of free radical formation in diabetic patients are scarce because of the inherent difficulties involved in measuring low concentrations of such highly reactivity and short-lived species in body fluids. Instead, surrogate markers are commonly employed in order to evaluate oxidative stress. In the present study, ROS production by granulocytes from patients with T1DM was significantly higher than in healthy subjects. This result is in accordance with that of Reis and cols. (11) who reported levels of ROS that were significantly elevated in 16 young patients with T1DM (age, 15.25 ± 3.83 years; duration of disease, 2.62 ± 2.24 years; HbA1c, 10.18 ± 2.5%) in comparison with their healthy counterparts. Ceriello and cols. (17) were able to demonstrate a significant increase in the production of superoxide anions (SOA) in 10 patients with T1DM, although the study was limited to adult subjects. On the other hand, Hsu and cols. (18) analyzed a group of 47 diabetic children (age, 12.7 ± 4.8 years; duration of disease, 4.94 ± 3.28 years; HbA1c, 9.22 ± 2.63%), and reported significantly higher levels of SOA compared with healthy individuals.

Increased levels of diacylglycerol (DAG) and the activation of PKC induced by hyperglycemia have also been associated with the initiation of diabetic complications. Phorbol esters are analogues of DAG and mimic the *in vitro* hyperglycemia effect of the glyceride by means of direct stimulation of PKC. In the present study, ROS generation in the presence of phorbol ester was increased both in patients with T1DM and in healthy subjects, but the enhancement was much more intense in diabetic patients (Figure 1). This suggests that hyperglycemia activates the expression of membrane PKC.

A significant increase in IL-6 levels between diabetic patients and healthy subjects was detected in the present study. This finding is consistent with other reports of significant increases in the concentration of IL-6 in patients with T1DM, but without micro-or macrovascular complications, in comparison with healthy individuals (19,20). Similarly, a recent SEARCH study (21) focusing on the association of between inflammation and obesity, dyslipidemia, and hyperglycemia in 553 young patients with T1DM, and 215 healthy individuals, found significantly higher levels of IL-6 in the diabetic group irrespective of weight or HbA1c concentration. In contrast, Erbagci and cols. (22) and Alexandraki and cols. (3) reported that there were no differences in IL-6 levels between children and adults with T1DM and their healthy counterparts.

The present study revealed no significant differences in the levels of inflammatory biomarkers between diabetic patients grouped according to progression of the disease (i.e., 0-10 years, 10-20 years, and > 20 years after diagnosis). However, when these groups were considered either individually or as a whole, such markers were significantly higher than in healthy subjects. The division by time elapsed after diagnosis is somewhat arbitrary, given that T1DM frequently lasts more than 5 years, and there is no literature studies that have evaluated inflammatory biomarkers in the intervals used in the present study. Because of this, it is not possible to draw a direct comparison of the present results with

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previously reported ones. However, Hsu and cols. (18) detected no significant differences between groups of children with T1DM when concentrations of SAO were compared in relation to disease duration (< 5 years *versus* > 5 years), even though levels of the biomarker were significantly higher in the diabetic children (grouped together) compared with healthy children. Erbagci and cols. (22) observed no differences in the levels of IL-6 between healthy children and those with T1DM, but when the diabetic children were divided according to time after diagnosis, IL-6 levels were higher in recently diagnosed (< 1 year) patients compared with long-standing (> 1 year) patients. In contrast, the SEARCH study found no differences in IL-6 concentrations between young patients with T1DM when grouped according to disease duration (< 1 year *versus* > 1 year after diagnosis) (21).

In the present study, no significant differences in plasma antioxidant status were observed between healthy subjects and patients with T1DM (considered together or grouped according to time elapsed since diagnosis). However, results revealed a slight, but not significant, increase in antioxidant status in diabetic patients who had been diagnosed for > 20 years. This increase was not attributable to the use of drugs, since none of the patients studied was receiving any medication other than insulin. To the best of our knowledge, only one published study is available reporting antioxidant concentrations (superoxide dismutase, glutathione peroxidase, glutathione reductase, and vitamins A, C, and E) in patients with T1DM grouped according to disease progression (< 5 years *versus* > 5 years after diagnosis), and it showed no between-group differences in the parameters (18).

The present study revealed that inflammatory biomarkers were not correlated with glycemic control (i.e., fasting glucose and HbA1c) or with any of the clinical and biochemical parameters studied. The possible correlation between inflammatory biomarkers and glycemic control is somewhat controversial. Acute hyperglycemia and poor glycemic control during the early stages of diabetes has been associated with increased inflammation in children with T1DM (3), but it is unclear whether chronic hyperglycemia, caused by long-established diabetes, is associated with inflammation. Thus, according to a study of 22 children with T1DM, Rosa and cols. (23) reported that acute hyperglycemia was associated with increased levels of IL-6, IL-4, and IL-1, and these elevated levels persisted for at least 2 h after glucose control. On the other hand, a number of studies have found no such associations (16,24,25). The lack of correlation between inflammatory biomarkers and levels of fasting glucose or HbA1c may suggest that, once triggered, inflammatory response can be modulated positively or negatively by parameters other than those studied, including, for example, glucose excursions (12). This hypothesis simply reinforces the complexity and multifactorial nature of T1DM.

The results obtained in the present study suggest that, during the first years after diagnosis of the disease, when the majority of patients do not present chronic complications, T1DM is characterized by a pro-inflammatory profile and absence of compensatory antioxidant response. However, this type of profile does not differ from that exhibited by patients with long-established T1DM in whom micro- and macrovascular complications are more prevalent. In other words, typical oxidative stress exists since early stages of the disease, and structural and functional changes involved in diabetic complications may start in the first years after diagnosis.

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