Enhanced Lipid Peroxidation and Platelet Activation in the Early Phase of Type 1 Diabetes Mellitus
Role of Interleukin-6 and Disease Duration

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Background—To investigate early events possibly related to the development of diabetic angiopathy, we examined whether 8-iso-prostaglandin F$_{2\alpha}$ (8-iso-PGF$_{2\alpha}$) formation, a marker of in vivo oxidant stress, is altered in different stages of type 1 diabetes (T1DM) and whether it correlates with the rate of thromboxane (TX) A$_2$ biosynthesis, a marker of in vivo platelet activation. We also investigated the relationship between inflammatory markers and F$_{2\alpha}$-isoprostane formation in this setting.

Methods and Results—A cross-sectional study was performed in 23 insulin-treated patients aged <18 years with new-onset T1DM (≤6 weeks, group A), matched for age and gender with 23 patients with stable disease (>1 year, group B). Urinary 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ were measured in all patients and in age- and gender-matched controls. Circulating interleukin-6 (IL-6), tumor necrosis factor-α, and C-reactive protein were also determined as markers of the inflammatory response. Fifteen of the 23 children in group A were reexamined after 12 months. Compared with either controls or group B, diabetic children in group A showed significantly higher levels of 8-iso-PGF$_{2\alpha}$, 11-dehydro-TXB$_2$, IL-6, tumor necrosis factor-α, and C-reactive protein. Statistically significant correlations between IL-6 and both 8-iso-PGF$_{2\alpha}$ (r=0.63, P<0.001) and 11-dehydro-TXB$_2$ (r=0.51, P<0.01) were observed. The 15 patients reexamined after 1 year showed a significant reduction in lipid peroxidation and platelet activation (P<0.02 and P<0.001, respectively), consistent with reduced levels of IL-6 and tumor necrosis factor-α.

Conclusions—These results demonstrate that enhanced lipid peroxidation and platelet activation represent early events in T1DM that are possibly related to an acute inflammatory response. These noninvasive indexes may help in further examining T1DM pathophysiology and monitoring pharmacological interventions to interfere with disease development and progression. (Circulation. 2003;107:3199-3203.)

Key Words: diabetes mellitus ■ inflammation ■ platelets

Diabetes mellitus is associated with the development of vascular complications that involve both the microcirculation and the macrocirculation. However, the mechanisms responsible for accelerated atherogenesis remain elusive.

Increased oxidative stress has been reported in diabetes mellitus. Both high glucose levels and protein glycation enhance LDL oxidation by metal ions, and these reactions also yield advanced glycosylation end (AGE) products. In fact, LDLs isolated from diabetics contain higher levels of AGE products and conjugated dienes and are more easily oxidized by copper than nondiabetic LDL. In addition, plasma from patients whose diabetes is poorly controlled has less antioxidant capacity and contains increased levels of thioarbituric acid-reactive substances and lipid hydroperoxides. We have reported that bioactive products of lipid peroxidation, such as the F$_{2\alpha}$-isoprostane 8-iso-prostaglandin (PG) F$_{2\alpha}$, are enhanced in the setting of diabetes mellitus; this abnormality was only partially reversible in association with improved glycemic control. 8-Iso-PGF$_{2\alpha}$ is a nonenzymatic oxidation product of arachidonic acid that is widely recognized as a reliable marker of lipid peroxidation both in vitro and in vivo. Enhanced lipid peroxidation, as reflected by increased 8-iso-PGF$_{2\alpha}$ excretion, has been reported previously in association with hypercholesterolemia, cigarette smoking, homozygous homocystinuria, renovascular hypertension, cystic fibrosis, and visceral obesity. Conversely, physical exercise has been associated with decreased 8-iso-PGF$_{2\alpha}$ formation.

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Moreover, 8-iso-PGF$_{2\alpha}$ induces vasoconstriction and can amplify agonist-induced platelet adhesion and aggregation.\textsuperscript{9,18,19} We have previously reported biochemical evidence of persistent platelet activation, as reflected by enhanced 11-dehydro-thromboxane (TX) B$_2$ excretion, in both type 1 and 2 diabetes mellitus\textsuperscript{8,20} and suggested that enhanced peroxidation of arachidonic acid to form biologically active isoprostanes may represent an important biochemical link between impaired glycemic control and persistent platelet activation.\textsuperscript{8}

Inflammatory cytokines may also trigger a similar chain of events leading to persistent platelet activation, as recently demonstrated in a different clinical paradigm, ie, visceral obesity.\textsuperscript{16} However, the relative contribution of impaired glycemic control, vascular complications, and inflammation in inducing persistent abnormalities in platelet function that characterize the diabetic state remains to be determined.

Development of type 1 diabetes is associated with T lymphocyte and macrophage activation determining a mononuclear cell infiltrate, a characteristic feature of the islet of Langerhans at diagnosis.\textsuperscript{21} Both activated T cells and macrophages operate and interact through the release of cytokines, which influence the type and magnitude of the immune response. It has been suggested that in predisposed individuals, a failure to switch from TH1 to TH2 dominance is an important factor in diabetes development,\textsuperscript{22} paralleled by a rise in the serum levels of a wide array of the TH1 subset of cytokines, including tumor necrosis factor-\alpha (TNF-\alpha), interleukin (IL)-1\beta, and IL-6, which may impair endothelial cell function.\textsuperscript{23,24} Therefore, the observation of elevated systemic IL-6 and TNF-\alpha levels in newly diagnosed cases of type 1 diabetes mellitus suggests activation of the inflammatory-immune response at early stages of the disease.\textsuperscript{25}

In the present study, we speculated that the early rise of TH1 macrophage-derived cytokines and the accompanying inflammatory response could induce increased oxidant stress, with enhanced generation of biologically active isoeicosanoids, and that these compounds could in turn trigger platelet activation in the early stages of type 1 diabetes mellitus. Therefore, we investigated whether 8-iso-PGF$_{2\alpha}$ formation is altered in newly diagnosed children and adolescents and whether it correlates with the rate of TXA$_2$ biosynthesis. Moreover, we investigated the relationship between inflammatory mediators and F$_2$-isoprostane formation both at diagnosis and after 1-year follow-up to test the reversibility of these changes with waning of the inflammatory response.

## Methods

### Patients

Forty-six young diabetic patients attending the Pediatric Diabetes Clinic, Division of Pediatrics, University of Chieti, Italy, were examined on several occasions. A cross-sectional study was performed in a group of 23 newly (<6 weeks) diagnosed patients (14 females and 9 males aged <18 years [mean±SD, 10±3 years]; group A) matched for age and gender with 23 patients (14 females and 9 males aged 12±4 years) with disease duration >1 year (group B). All had type 1 diabetes mellitus as defined in accordance with the criteria of the American Diabetes Association\textsuperscript{26} and were taking insulin treatment (0.8 to 1.2 U·kg$^{-1}$·d$^{-1}$). Median diabetes duration (defined on the basis of the first injection of insulin) was 4 (range 3 to 6) weeks in group A and 144 (range 62 to 336) weeks in group B. None of the children had clinical evidence of retinopathy, as assessed by routine ophthalmologic examination and stereoscopic fundus photography, or nephropathy, as reflected by microalbuminuria or macroalbuminuria. None were taking nonsteroidal anti-inflammatory drugs, low-dose aspirin, or vitamin supplements. Twenty-three age- and gender-matched healthy children were also studied. Fifteen of the 23 newly diagnosed diabetic patients were reexamined after 12 months. Eight subjects were lost to follow-up: 3 were referred to another diabetic center, 4 females had menarche, and 1 had newly diagnosed celiac disease.

Informed consent was obtained from each subject participating in the study and his or her parents. The protocol was approved by the local ethics committee. The baseline characteristics of patients and control subjects are detailed in Table 1.

### Biochemical Measurements

Peripheral venous blood samples were withdrawn into tubes containing 3.8% sodium citrate (9 mL of blood to 1 mL of sodium citrate). Plasma was prepared by centrifugation (2000g for 20 minutes), separated into

## TABLE 1. Clinical Characteristics of Healthy Subjects and Type 1 Diabetic Patients According to Disease Duration

<table>
<thead>
<tr>
<th>Variables</th>
<th>Healthy Subjects (n=23)</th>
<th>New Onset (n=23)</th>
<th>Stable &gt;1 Year (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, female/male</td>
<td>14/9</td>
<td>14/9</td>
<td>14/9</td>
</tr>
<tr>
<td>Age, y</td>
<td>11±3</td>
<td>10±3</td>
<td>12±4</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dL</td>
<td>88±9</td>
<td>193±81</td>
<td>175±89</td>
</tr>
<tr>
<td>Diabetes duration, wk</td>
<td>...</td>
<td>...</td>
<td>9.1±2.5</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>...</td>
<td>13.2±3.1</td>
<td>14.9±2.3</td>
</tr>
<tr>
<td>AGE products, U/mL</td>
<td>...</td>
<td>0.48 (0.26–0.66)</td>
<td></td>
</tr>
<tr>
<td>Plasma TNF-\alpha, pg/mL</td>
<td>0.48 (0.26–0.66)</td>
<td>3.50 (2.40–5.20)</td>
<td></td>
</tr>
<tr>
<td>Plasma CRP, mg/L</td>
<td>0.32 (0.24–0.44)</td>
<td>3.20 (1.90–4.00)</td>
<td></td>
</tr>
<tr>
<td>Plasma IL-6, pg/mL</td>
<td>0.32 (0.20–0.40)</td>
<td>2.00 (1.30–3.20)</td>
<td></td>
</tr>
</tbody>
</table>

*F indicates ANOVA; H, Kruskal-Wallis test. P value represents modified least squares difference (Bonferroni) test. Data are presented as mean±SD or median (25th–75th percentile).

Healthy subjects vs new-onset type 1 diabetic patients; new-onset vs stable >1 year type 1 diabetic patients; stable >1 year type 1 diabetic patients vs healthy subjects.
алиоты, and stored at −80°C. TNF-α and IL-6 plasma levels were measured with ELISA kits (R&D Systems Europe Ltd). Intra-assay and interassay coefficients of variation were <9%.

Plasma C-reactive protein (CRP) levels were measured with a highly sensitive immunoassay.²⁷ AGE products were measured by an immunochemical method, as described previously.²⁸

One AGE product unit was defined as the competitive activity of 1 μg of AGE-BSA standard.²⁸ The serum concentration of AGE products was corrected for total protein concentration in each serum sample with the following equation: AGE (μM/L) = (sample protein concentration/mean protein concentration of all sera measured).

Fasting plasma glucose was measured by the glucose oxidase method. Hemoglobin (Hb) A₁c was determined by automated high-performance liquid chromatography.²⁹

**Urinary Eicosanoid Assays**

Urinary 8-iso-PGF₂α and 11-dehydro-TXB₂ were measured by previously described radioimmunoassay methods.³⁰,³¹ Measurements of urinary eicosanoid metabolites by these radioimmunoassays have been validated with different antisera and by comparison with gas chromatography/mass spectrometry, as detailed elsewhere.³⁰,³¹

**Statistical Analysis**

Statistical analyses were performed by χ² statistics or Fisher’s exact test (if n<5) for independence and by appropriate t test. Correlations were analyzed by the Spearman rank correlation test. Multiple linear regression analysis was performed to assess independent predictors of 8-iso-PGF₂α excretion. Data are presented as mean±SD and 95% CI. TNF-α, CRP, and IL-6 values are expressed as median and interquartile range because they showed skewed distribution. Only 2-tailed probabilities were used for testing statistical significance. Probability values <0.05 were regarded as statistically significant. All calculations were made with the computer program Stat-View II (Abacus Concepts).

**Results**

Newly diagnosed diabetic patients had significantly higher urinary 8-iso-PGF₂α excretion than patients with longstanding disease (P<0.05; Figure 1). All patients with new-onset diabetes and 70% of those with stable disease had excretion rates in excess of 2 SD above the healthy control mean.

Urinary 11-dehydro-TXB₂ excretion was also significantly higher in newly diagnosed diabetic patients than in patients with stable disease (Figure 1). Seventy-three percent of newly diagnosed patients and 39% of patients with stable disease had metabolite excretion rates in excess of 2 SD above the healthy control mean. A statistically significant linear correlation was found between 8-iso-PGF₂α and 11-dehydro-TXB₂ excretion rates in both groups of diabetic patients (r=0.73, P<0.0001 and r=0.70, P<0.001, respectively).

As detailed in Table 1, circulating levels of TNF-α, IL-6, and CRP were also increased in new-onset diabetic subjects compared with either healthy subjects or patients with longer disease duration. The latter group also showed significantly higher circulating levels of these inflammatory markers than normal subjects (Table 1).

To further define the relationship between F₂-isoprostane biosynthesis and indexes of inflammation, metabolic control, and disease duration, multiple regression analysis was performed with baseline 8-iso-PGF₂α excretion as the dependent variable. These results are detailed in Table 2 and show that serum levels of IL-6 (P=0.027) and disease duration (P=0.025) predicted 8-iso-PGF₂α excretion rate (Figure 2) independently of CRP, TNF-α, and HbA₁c in newly diagnosed patients.

Fifteen of the 23 newly diagnosed diabetic patients were reexamined after 1 year to test the reversibility of these changes with waning of the inflammatory response. Urinary 8-iso-PGF₂α excretion was significantly (P<0.02) decreased in these 15 patients compared with the time of diagnosis (Figure 3). Urinary 11-dehydro-TXB₂ excretion was also significantly (P<0.001) decreased (Figure 3). Excretion rates of both metabolites after 1 year were indistinguishable from those measured in the group of diabetic patients with disease duration >1 year. The serum levels of IL-6 (P<0.002) and

**TABLE 2. Multiple Regression Analysis of Urinary 8-iso-PGF₂α Excretion in Relation to Indexes of Inflammation, Metabolic Variables, and Disease Duration**

<table>
<thead>
<tr>
<th>Variable</th>
<th>β-Coefficient</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.08</td>
<td>0.18</td>
<td>0.677</td>
</tr>
<tr>
<td>Body mass index</td>
<td>−0.12</td>
<td>0.22</td>
<td>0.582</td>
</tr>
<tr>
<td>HbA₁c</td>
<td>−0.28</td>
<td>0.24</td>
<td>0.259</td>
</tr>
<tr>
<td>AGEs</td>
<td>0.20</td>
<td>0.18</td>
<td>0.286</td>
</tr>
<tr>
<td>Plasma TNF-α</td>
<td>−0.27</td>
<td>0.29</td>
<td>0.367</td>
</tr>
<tr>
<td>Plasma CRP</td>
<td>0.05</td>
<td>0.21</td>
<td>0.814</td>
</tr>
<tr>
<td>Disease duration</td>
<td>−0.51</td>
<td>0.20</td>
<td>0.025</td>
</tr>
<tr>
<td>Plasma IL-6</td>
<td>0.73</td>
<td>0.29</td>
<td>0.027</td>
</tr>
</tbody>
</table>
TNF-α (P<0.04) were significantly decreased after 1 year compared with the time of diagnosis, whereas CRP displayed a nonsignificant trend in the same direction (Table 3).

Discussion

Our results demonstrate that enhanced lipid peroxidation and platelet activation represent early events in the development of type 1 diabetes mellitus in children and adolescents. We observed that patients with newly diagnosed diabetes had significantly increased urinary excretion of both 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ and higher plasma levels of a number of inflammatory markers; in some of these patients, oxidative stress and platelet activation were reduced after 1 year, coincident with a fall in the systemic levels of IL-6 and TNF-α. Thus, it appears that biochemical signals of oxidative stress and platelet activation can be appreciated early at the onset of diabetes mellitus and that their variable intensity is driven, at least in part, by IL-6 production and disease duration.

An inflammatory response is frequently observed in the initial phase of type 1 diabetes mellitus. The finding that those patients with the shortest duration of disease and with the highest IL-6 levels had the highest rates of in vivo lipid peroxidation and platelet activation is consistent with the hypothesis that in children with type 1 diabetes, the early increase in oxidative stress and platelet activation may be associated with inflammatory events that precede clinical manifestation of the disease. Once established, oxidative stress may sustain a vicious circle, because it has been shown that hydrogen peroxide induces the IL-6 promoter by activating nuclear factor-kB through nuclear factor-kB–inducing kinase. Consistent with earlier findings, type 1 diabetics showed higher rates of in vivo lipid peroxidation and platelet activation than age- and gender-matched healthy subjects. However, the rate of eicosanoid metabolite excretion was significantly different between the 2 groups of diabetic children, consistent with the influence of disease duration on the rate of lipid peroxidation. In addition to triggering platelet activation via enhanced formation of bioactive isoprostanes, as suggested by the results of the present study, IL-6 could induce a prothrombotic state by enhancing thrombin-induced platelet activation. Because there may be a multiplicity of inflammatory mediators reflecting the individual host response to a local immune reaction in the pancreatic β-cells, it is perhaps not surprising that IL-6 variability only explains a small percentage of the variability in 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ excretion. Other inflammatory mediators or the immune reaction per se may represent the main triggers of enhanced lipid peroxidation and platelet activation, as suggested by the consistent fall in 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ recorded 1 year after the onset of the disease, at a time when the immune/inflammatory reaction presumably had waned.

We conclude that enhanced lipid peroxidation and platelet activation represent early events in type 1 diabetes mellitus that are possibly related to an acute inflammatory response. Furthermore, isoprostane biosynthesis may represent a sensitive marker of the immune/inflammatory reaction with which to further examine the pathophysiology of type 1 diabetes.
mellitus and monitor pharmacological interventions aimed at interfering with disease development and progression.

Acknowledgments

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