In Vivo Formation of 8-Iso-Prostaglandin F\textsubscript{2\alpha} and Platelet Activation in Diabetes Mellitus

Effects of Improved Metabolic Control and Vitamin E Supplementation

Giovanni Davi, MD; Giovanni Ciabattoni, MD; Agostino Consoli, MD; Andrea Mezzetti, MD; Antonio Falco, MD; Stella Santarone, MD; Elsa Pennese, MD; Ester Vitacolonna, MD; Tonino Bucciarelli, MD; Fabrizio Costantini, MD; Fabio Capani, MD; Carlo Patrono, MD

Background—Diabetes mellitus (DM) is associated with enhanced lipid peroxidation and persistent platelet activation. We tested the hypothesis that the in vivo formation of the F\textsubscript{2}-isoprostane 8-iso-prostaglandin (PG)F\textsubscript{2\alpha}, a bioactive product of arachidonic acid peroxidation, is enhanced in DM and contributes to platelet activation.

Methods and Results—Urine samples were obtained from 85 diabetic patients and 85 age- and sex-matched healthy subjects for measurement of immunoreactive 8-iso-PGF\textsubscript{2\alpha} and 11-dehydro-thromboxane B\textsubscript{2} (TXM), an in vivo index of platelet activation. Sixty-two had non–insulin-dependent (NID)DM, and 23 had insulin-dependent (ID) DM. Vitamin E supplementation, metabolic control, and cyclooxygenase inhibitors were used to investigate the mechanisms of formation of 8-iso-PGF\textsubscript{2\alpha} in this setting. Urinary 8-iso-PGF\textsubscript{2\alpha} excretion was significantly higher (\textit{P} < 0.0001) in NIDDM patients (419 ± 208 pg/mg creatinine; range 160 to 1014) than in age-matched control subjects (208 ± 92; 41 to 433). Urinary 8-iso-PGF\textsubscript{2\alpha} was linearly correlated with blood glucose and urinary TXM. 8-iso-PGF\textsubscript{2\alpha} excretion was also significantly (\textit{P} = 0.0001) higher in IDDM patients (400 ± 146; 183 to 702) than in control subjects (197 ± 69; 95 to 353). Vitamin E supplementation (600 mg/d for 14 days) was associated with a statistically significant reduction in both urinary 8-iso-PGF\textsubscript{2\alpha} (by 37%) and TXM (by 43%) in 10 NIDDM patients. Improved metabolic control was associated with a significant (\textit{P} < 0.0001) reduction in 8-iso-PGF\textsubscript{2\alpha} and TXM excretion by 32% and 41%, respectively, in 21 NIDDM patients. 8-iso-PGF\textsubscript{2\alpha} was unchanged after 2-week dosing with aspirin and indobufen despite profound suppression of TXM excretion.

Conclusions—We conclude that DM is associated with increased formation of F\textsubscript{2}-isoprostanes, as a correlate of impaired glycemic control and enhanced lipid peroxidation. This may provide an important biochemical link between impaired glycemic control and persistent platelet activation. These results provide a rationale for dose-finding studies of antioxidant treatment in diabetes. (\textit{Circulation}. 1999;99:224-229.)

Key Words: diabetes mellitus ■ platelets ■ lipids ■ thromboxane ■ antioxidants

There is a well-established association of diabetes mellitus with the development of atherosclerosis and its thromboembolic complications and with the occurrence of disorders of the microvasculature.\textsuperscript{1} However, the mechanism(s) responsible for accelerated atherogenesis remain elusive. Altered lipoprotein levels; changes in lipoprotein composition, possibly affecting low-density lipoprotein (LDL) binding to its receptors; and reduced LDL clearance resulting from impaired receptor recognition of glycated LDL have been described in diabetic patients (reviewed in Reference 2). Moreover, both high glucose levels and protein glycation enhance LDL oxidation by metal ions, and these reactions also yield advanced glycosylation end (AGE) products.\textsuperscript{2,3} In fact, LDLS isolated from non–insulin-dependent diabetics (NIDDM) contain higher levels of AGE products and conjugated dienes and are more easily oxidized by copper than native LDL.\textsuperscript{4} In addition, plasma from patients whose insulin-dependent diabetes mellitus (IDDM) is poorly controlled has less antioxidant capacity.\textsuperscript{5}

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Recently, a series of bioactive prostaglandin (PG) F\textsubscript{2}-like compounds (isoprostanes) has been discovered,\textsuperscript{6} which are produced from arachidonic acid through a nonenzymatic process of lipid peroxidation, catalyzed by oxygen free radicals on cell membranes and LDL particles (reviewed in
Reference 7). Among these products, of particular importance is 8-isoprostane, which induces vasoconstriction and modulates the function of human platelets.2–11

F₂-isoprostanes can be reliably measured in both plasma and urine12,13 and have been shown to be increased in association with advanced age,13 hypercholesterolemia,14 and cigarette smoking.15,16

We have previously reported biochemical evidence of persistent platelet activation, as reflected by enhanced 11-dehydro-TXB₂ (TXM) excretion, in NIDDM patients with macrovascular complications and shown that this abnormality was partially reversible in association with improved glycemic control.17 We speculated that increased oxidant stress in diabetes could induce enhanced generation of 8-isoprostane and other biologically active iso-eicosanoids and that these compounds could in turn contribute to platelet activation in this setting. Therefore, in the present study, we investigated whether 8-isoprostane formation is altered in NIDDM patients compared with age-matched nondiabetic subjects and whether it correlates with the rate of TXB₂ biosynthesis. We also measured F₂-isoprostane formation in IDDM patients as compared with their age-matched control subjects to assess the relative contribution to oxidant stress of the diabetic metabolic abnormality per se, vis-à-vis the macrovascular complications often accompanying NIDDM. Moreover, we examined the effects of 2 interventions potentially capable of reducing oxidant stress, that is, improvement in metabolic control and vitamin E supplementation, by assessing time-related changes in urinary 8-isoprostane and TXM excretion in NIDDM.

The results of the present study suggest 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 in this setting.

Methods

Subjects

Eighty-five patients with diabetes mellitus were studied on several occasions between May 1995 and December 1997. Sixty-two had NIDDM and 23 had IDDM, as defined in accordance with the criteria of the American Diabetes Association.18 Sixty-two and 23 patients, respectively, were also studied. The baseline characteristics of patients and control subjects are detailed in Table 1.

Of the 62 NIDDM patients, 20 patients had a history or physical examination positive for evidence of macrovascular complications. Twelve patients had stable angina pectoris or had had a myocardial infarction, and 8 had peripheral vascular disease. Patients with coronary heart disease were in a stable phase. Patients with peripheral vascular disease were in Fontaine stage II (intermittent claudication, ankle-arm pressure index of <0.85, and no resting pain), with a constant level of pain while walking. At the time of the study, NIDDM patients were being treated by diet alone (4 patients), insulin alone (6 patients), or by diet plus oral hypoglycemic agents (metformin and/or sulfonylureas) (43 patients); in 9 patients, insulin was added to the oral hypoglycemic agents. Twenty-four patients had arterial hypertension, defined as current systolic/diastolic blood pressure >140/90 mm Hg. Twenty-six patients were hypercholesterolemic (blood cholesterol level >240 mg/dL).

None of the 23 IDDM patients had a history or physical examination positive for evidence of macrovascular complications. Nine patients had diabetic retinopathy as determined by direct ophthalmoscopy through a dilated pupil. Only 1 IDDM patient was hypertensive as defined above. At the time of the study, all IDDM patients were being treated with insulin therapy.

Patients with renal insufficiency or proteinuria (by serum creatinine levels and urinalysis), altered hepatic function (by liver enzymes), or alcohol abuse (by clinical history and laboratory measurements) were excluded. Both diabetic patients and healthy subjects were selected for being nonsmokers at the time of study to eliminate a potential confounder.

Informed consent was obtained from each participating subject, and the protocol was approved by the ethical committee of the University of Chieti Medical School.

Design of the Studies

In the first study, a cross-sectional comparison of urinary 8-isoprostane and TXM, a major enzymatic metabolite of TXA₂, was performed between patients and control subjects. All the subjects were studied as outpatients after a 12-hour fast. Each patient performed an overnight urine collection, immediately before blood sampling. Urine samples were added with the antioxidant 4-hydroxy-tempo (1 mmol/L) (Sigma Chemical Co) and stored at −20°C until extraction.

Because small amounts of 8-isoprostane can be formed by human platelets and monocytes through a cyclooxygenase-dependent mechanism,2 a second study was performed to evaluate whether inhibition of cyclooxygenase activity had any influence on 8-isoprostane excretion in diabetes. For this purpose, 6 of the 62 NIDDM patients (3 women and 3 men; 56 to 75 years of age) were given 50 mg acetylsalicylic acid once daily and 200 mg indobufen (a reversible cyclooxygenase inhibitor) twice daily, each drug for 7 days in 2 successive weeks, according to a randomized sequence. These patients collected overnight urine samples before dosing and on the last day of each treatment for measurement of 8-isoprostane and TXM excretion.

To assess the potential influence of improved metabolic control on F₂-isoprostane formation and platelet activation, a third study was performed in 21 NIDDM patients (13 women and 8 men; 39 to 75 years of age). At the time of the cross-sectional study, these 21 NIDDM patients were in poor metabolic control (fasting blood glucose ≥200 mg/dL, glycohemoglobin [HbA₁c] >9%) despite taking oral antidiabetic agents for months. These patients were examined twice weekly over a 4-week period for blood glucose monitoring, and oral antidiabetic therapy was adjusted accordingly and/or insulin therapy was instituted to achieve improved metabolic control. Throughout the study, patients followed an isocaloric diet that provided 50% of calories as carbohydrates, 30% as fat, and 20%

| TABLE 1. Clinical Characteristics of Diabetic Patients and Healthy Subjects |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| Variables                  | NIDDM (n=62)    | Healthy (n=62)  | IDDM (n=23)     | Healthy (n=23)  |
| Sex, female/male           | 31/31           | 31/31           | 6/17            | 6/17            |
| Age, y                     | 61±9            | 57±9            | 40±14           | 41±12           |
| Diabetes duration, y (range)| 11±9            | ...             | 16±10           | ...             |
| Fasting blood glucose, mg/dL| 209±99         | ND              | 161±68          | ND              |
| HbA₁c, %                   | 8.7±2.0         | ND              | 7.0±1.4         | ND              |
| Hypertension, %            | 39              | 0               | 4               | 0               |
| Hypercholesterolemia, %    | 42              | 0               | 30              | 0               |
| Macrovascular complications, % | 32             | 0               | 0               | 0               |
| Microvascular complications, % | 18             | 0               | 35              | 0               |

ND indicates not determined.
as protein. The level of dietary cholesterol was \( \sim 0.3 \) g per day. Physical activity was encouraged, and patients were instructed to walk at least 30 minutes after each meal. No patient experienced a hypoglycemic reaction during the study period. Blood and overnight urine samples were obtained before and at the end of this intensive monitoring and treatment program for determination of fasting glucose and \( \text{HbA}_{1c} \) levels and 8-iso-PGF\(_{2\alpha}\) and TXM excretion, respectively.

To investigate the short-term effects of antioxidant intervention on urinary 8-iso-PGF\(_{2\alpha}\) and TXM excretion, vitamin E was given to 10 (6 women and 4 men; 42 to 69 years of age) of the 62 NIDDM patients. They were given 600 mg/d \( \alpha \)-tocopherol acetate (Evion) daily for 2 weeks after a baseline evaluation. This dose of vitamin E is \( \sim 60 \) fold higher than the recommended daily dietary allowance. Before and after vitamin E supplementation, they collected an overnight urine sample for measurement of 8-iso-PGF\(_{2\alpha}\) and TXM and had a fasting blood sample drawn for lipid levels, plasma and LDL vitamin E, and oxidation of isolated LDL in vitro.

**Urinary Eicosanoid Assays**

Urinary 8-iso-PGF\(_{2\alpha}\) and TXM were measured by previously described radioimmunoassay methods.\textsuperscript{13,19} Measurements of urinary 8-iso-PGF\(_{2\alpha}\) and TXM by these radioimmunoassays have been validated with the use of different antisera and by comparison with gas chromatography/mass spectrometry, as detailed elsewhere.\textsuperscript{13,19}

**Lipid Measurements**

All blood samples for lipid studies were drawn into EDTA (1 mg/mL and separated within 1 hour after sampling. Total, LDL and high-density lipoprotein cholesterol, triglycerides, and vitamin E were determined as previously described.\textsuperscript{14} Aliquots of the plasma in EDTA, immediately after separation, were stored at \(-80\) °C until LDL isolation. LDL was isolated by single vertical spin density gradient ultracentrifugation, and LDL protein, cholesterol, and vitamin E were determined as previously described.\textsuperscript{14} To induce oxidation, LDL (0.2 mg cholesterol/mL) was incubated with 5 mmol/L CuSO\(_4\) in PBS, pH 7.4, at 37 °C. The formation of conjugated dienes was determined spectrophotometrically.\textsuperscript{14}

**Clinical Laboratory Measurements**

Fasting plasma glucose was measured by the glucose oxidase method. \( \text{HbA}_{1c} \) level was measured by automated high-performance liquid chromatography.

**Statistical Analysis**

The data were analyzed by nonparametric methods to avoid assumptions about the distribution of the measured variables. ANOVA was performed with the Kruskal-Wallis method. Subsequent pairwise comparisons were made with the Mann-Whitney \( U \) test. The differences between baseline and posttreatment values were analyzed with the Wilcoxon signed rank test. Moreover, the association of eicosanoid measurements with other biochemical parameters was assessed by the Spearman rank correlation test. All values are reported as mean \( \pm \) SD.

**Results**

Urinary 8-iso-PGF\(_{2\alpha}\) excretion was significantly \( (P=0.0001) \) higher in NIDDM patients \((419 \pm 208 \) pg/mg creatinine; mean \( \pm \) SD, \( n=62 \)) than in age-matched healthy subjects \((208 \pm 92 \) pg/mg creatinine) (Figure 1). Twenty-six of the 62 NIDDM had blood cholesterol \( \geq 240 \) mg/dL \((292 \pm 37 \) and abnormal LDL cholesterol levels \((189 \pm 38 \) mg/dL). The urinary excretion of 8-iso-PGF\(_{2\alpha}\) was not significantly higher in these hypercholesterolemic patients than in the 36 normocholesterolemic NIDDM patients \((438 \pm 178 \) vs \( 401 \pm 227 \) pg/mg creatinine). Similarly, no statistically significant differences in urinary 8-iso-PGF\(_{2\alpha}\) were found between hyper- and normotensive and normotensive NIDDM patients \((429 \pm 219 \) vs \( 412 \pm 203 \) pg/mg creatinine) nor between those with and those without macrovascular complications \((459 \pm 233 \) vs \( 400 \pm 195 \) pg/mg creatinine).

Consistent with previous findings, NIDDM patients had significantly enhanced TXM excretion versus that in control subjects \((1103 \pm 1068 \) vs \( 415 \pm 244 \) pg/mg creatinine; \( P=0.0001) \). A statistically significant correlation was found between TXM and 8-iso-PGF\(_{2\alpha}\) excretion in NIDDM patients \((r=0.39, \ P=0.0023)\).

The urinary excretion of 8-iso-PGF\(_{2\alpha}\) was also increased in the group of IDDM patients \((400 \pm 146 \) pg/mg creatinine; \( n=23 \)) as compared with their age-matched control subjects \((197 \pm 69 \) pg/mg creatinine; \( P=0.0001) \) (Figure 2).

**Effects of Cyclooxygenase Inhibition**

Two structurally unrelated cyclooxygenase inhibitors, aspirin and indobufen, were used to investigate the mechanism(s) of F\(_2\)-isoprostane formation in NIDDM. As shown in Figure 3, urinary 8-iso-PGF\(_{2\alpha}\) excretion was largely unaffected during 2 successive weeks of cyclooxygenase inhibition achieved with either agent, despite normalization of TXM excretion. This
finding is consistent with a noncyclooxygenase mechanism of F₂-isoprostane formation as characterized in other clinical settings.Ä This study also allowed assessing the reproducibility of 8-iso-PGF₂₅α excretion in NIDDM on the basis of 3 different urine collections performed over a 2-week period in 6 patients. The intrasubject coefficient of variation of these measurements averaged 19±11%.

Effect of Metabolic Control

Twenty-one NIDDM patients who had not achieved adequate metabolic control (fasting blood glucose >200 mg/dL and HbA₁c >9%) despite oral antidiabetic therapy were subjected to intensive monitoring and treatment over 4 weeks, and blood and urine samples were obtained before and after improved metabolic control. At the end of this period, HbA₁c fell from 9.9±1.3% to 7.5±1.0% (P<0.0001), and fasting blood glucose was significantly reduced from 306±118 to 159±47 mg/dL (P<0.0001). Improvement in metabolic control was associated with a statistically significant reduction in urinary 8-iso-PGF₂₅α and TXM, by 37% and 43%, respectively. A statistically significant correlation was found between the 2 sets of measurements (r=0.582; P=0.0112). Individual values of 8-iso-PGF₂₅α fell within the normal range in all NIDDM patients treated with vitamin E.

Discussion

We have previously demonstrated enhanced TXA₂ biosynthesis in NIDDM patients and provided evidence for its platelet origin and its reduction in response to tight metabolic control.Ä Furthermore, a recently completed study suggests that the metabolic disorder rather than the attendant vascular disease is responsible for persistent platelet activation in NIDDM. In the present report, we performed a series of studies to test the hypothesis that persistent platelet activation may, at least in part, be related to enhanced formation of biologically active products of arachidonic acid peroxidation. Previous evidence for enhanced lipid peroxidation in diabetes mellitus is largely indirect and based on crude measurements of lipid oxidation products in plasma.Ä 

Table 2. Concentrations of Vitamin E in Plasma, Vitamin E Content of LDL, and Lag Time for LDL Oxidation in NIDDM Patients Before and After Vitamin E Supplementation

<table>
<thead>
<tr>
<th>Dose of Vitamin E, mg/d</th>
<th>Plasma Vitamin E, μmol/L</th>
<th>LDL Vitamin E, μg/mg LDL Cholesterol</th>
<th>Lag Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32±9</td>
<td>3.6±0.8</td>
<td>59±13</td>
</tr>
<tr>
<td>600</td>
<td>60±18*</td>
<td>5.1±1.2</td>
<td>81±21*</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=10).

*Statistically significant (P<0.01) difference between the 2 sets of measurements.
susceptibility of the patient’s LDL to oxidation in vitro. Our study has used the urinary excretion of the F2-isoprostane 8-iso-PGF2α, as a marker of in vivo lipid peroxidation.

We found that the formation and urinary excretion of 8-iso-PGF2α was abnormally elevated in the vast majority of a relatively large group of NIDDM patients carefully characterized for other variables potentially influencing lipid peroxidation. Thus we excluded the contribution of advanced age by adequate age-matching of individual patients and control subjects and of cigarette smoking by only recruiting nonsmokers into the study. Similarly, differences in 8-iso-PGF2α formation between diabetic patients and healthy subjects could not be accounted for by the presence of macrovascular complications, arterial hypertension, or hypercholesterolemia.

We found a highly significant correlation between blood glucose and urinary 8-iso-PGF2α, suggesting that lipid peroxidation may be, at least in part, related to the determinants of glycemic control. This observation is consistent with the in vitro findings of Natarajan et al., who demonstrated enhanced formation and release of 8-iso-PGF2α by porcine vascular smooth muscle cells cultured under hyperglycemic conditions. Gopaul et al. previously reported increased plasma levels of esterified 8-iso-PGF2α in NIDDM that did not correlate with fasting glucose or with HbA1c. Whether the greater variability in plasma versus urinary measurements of this F2-isoprostane or the small sample size of the latter study contribute to this apparent discrepancy remains unanswered by the present study.

That impaired glycemic control rather than the attendant macrovascular complications is responsible for enhanced formation of F2-isoprostanes in NIDDM is also supported by the similar findings in IDDM patients (Figure 2). Catella-Lawson et al. have recently reported a trend toward increased urinary 8-iso-PGF2α excretion in a group of 18 diabetics, with statistically significant elevations in patients with diabetic ketoacidosis.

We further examined the relation between metabolic control and F2-isoprostane formation by studying 21 NIDDM patients with inadequate glycemic control, before and after intensive antidiabetic treatment, and closer monitoring. Reduced blood glucose levels were associated with a fall in urinary 8-iso-PGF2α excretion rates, the average extent of which showed a remarkably good fitting, with the linear relation between blood glucose and urinary 8-iso-PGF2α, as established in the whole group of NIDDM patients under baseline conditions. Improvement of metabolic control in these patients was accompanied by a statistically significant reduction in TXM excretion by 41% as compared with 45% in the previous study carried out with a similar protocol. On the basis of these findings, it is tempting to speculate that changes in the rate of arachidonate peroxidation to form biologically active iso-eicosanoids, such as 8-iso-PGF2α, may represent an important biochemical link between altered glycemic control, oxidant stress, and platelet activation in NIDDM.

That changes in F2-isoprostane formation are not merely a consequence of persistent platelet activation is clearly indicated by the study with 2 structurally unrelated cyclooxygenase inhibitors. Thus 70% reduction in TXM excretion—corresponding to complete normalization of TXA2 biosynthesis—was not accompanied by any detectable change in urinary 8-iso-PGF2α excretion. This finding is consistent with the noncyclooxygenase mechanism of formation of 8-iso-PGF2α in other clinical settings characterized by enhanced lipid peroxidation.

Having established that formation of 8-iso-PGF2α is largely independent of cyclooxygenase activity, we set out to assess the reversibility of its increase in response to vitamin E supplementation. We choose a pharmacological dose of vitamin E, 600 mg daily, based on the results of a similar study performed in hypercholesterolemic patients demonstrating dose-dependent reduction in F2-isoprostane formation in response to short-term vitamin E supplementation. Similar to the results obtained in hypercholesterolemic patients, we found virtually complete normalization of 8-iso-PGF2α excretion in NIDDM in the present study. Moreover, changes in F2-isoprostane formation were accompanied by similar reductions in TXM excretion, consistent with a cause-and-effect relation between enhanced lipid peroxidation and persistent platelet activation in this setting.

Concentrations of 8-iso-PGF2α in the range of 1 nmol/L to 1 μmol/L induce a dose-dependent increase in platelet shape change, calcium release from intracellular stores, and inositol phosphates. Moreover, 8-iso-PGF2α increases platelet adhesion and reduces the antiadhesive and antiaggregatory effects of nitric oxide. Furthermore, 8-iso-PGF2α causes dose-dependent, irreversible platelet aggregation in the presence of concentrations of collagen, ADP, arachidonic acid,
and PGH₂/TXA₂ analogues that when acting alone fail to aggregate platelets.⁷¹ Although platelet-active concentrations of 8-iso-PGF₂α, may not be achieved in circulating blood, it should be pointed out that this compound is but one of a series of biologically active iso-eicosanoids formed through a similar noncyclooxygenase mechanism of lipid peroxidation.⁷² We believe that our results have potential clinical implications for the prevention of atherothrombosis in diabetic patients. Given the relatively limited impact of conventional antiplatelet prophylaxis in this setting, the present findings suggest the opportunity of exploring combined preventive strategies based on the combination of low-dose aspirin and vitamin E supplementation. The urinary excretion of 8-iso-PGF₂α provides a noninvasive, reproducible biochemical end point for dose-finding studies of vitamin E supplementation.⁷³ This may help resolve the present uncertainty about the optimal dose of vitamin E and provide a more rational basis for dose selection for large-scale intervention trials.

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