# $F_2$ Isoprostane Is Already Increased at the Onset of Type 1 Diabetes Mellitus: Effect of Glycemic Control

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**Much evidence has suggested that oxidative stress (OS) may play a role in the pathogenesis of diabetic complications. However, the relationship between hyperglycemia and OS is inconsistent in diabetic clinical studies. The aim of this study was to evaluate the effect of normalization of blood glucose levels on urinary 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) excretion at the onset of type 1 diabetes. We studied 14 type 1 diabetic patients (50% males; mean age, 24.3 ± 4.9 years) and 14 control subjects matched by age and body mass index. A 24-hour urine collection was performed to determine 8-epi-$PGF_{2\alpha}$ as an integrated index of OS production at baseline, before starting insulin therapy, and 16 weeks later. Insulin treatment induced a significant reduction in glycosylated hemoglobin ($HbA_{1c}$) (from 11.5% to 5.4% $P = .0001$), triglycerides (from 1.0 to 0.8 mmol/L, $P = .002$), and an increase in high-density lipoprotein (HDL)-cholesterol levels (from 1.1 to 1.5 nmol/L, $P = .01$) at week 16. This improvement in metabolic control was associated with a statistically significant reduction in 8-epi-$PGF_{2\alpha}$ values (from 92.0 ± 41.5 to 66.9 ± 28.9 pg/mg urinary reatinine excretion, $P = .015$), although compared with the control group, 8-epi-$PGF_{2\alpha}$ values remained higher in diabetic patients (66.9 ± 28.9 *v* 39.1 ± 13.8 pg/mg creatinine, $P = .004$). Enhanced OS is present in early clinical phases of type 1 diabetes, and the amelioration in metabolic control is associated with improvement in this pathogenic pathway.**



LIPID PEROXIDATION (LP) is the main marker of oxidative stress (OS), a pathogenic pathway strongly related to the development of diabetic complications. Under diabetic conditions, OS may be due to many factors, such as increased reactive oxygen species (ROS) production via glucose autooxidation, nonenzymatic protein glycation, oxidative DNA damage, decreased antioxidant status, and consequently, ineffective scavenging of ROS.[1-3] Experimental data have clearly demonstrated that hyperglycemia mediates the deleterious effects of OS. However, the relationship between glycemic control and OS is inconsistent in diabetic clinical studies.[4-5] This discrepancy is probably related to the lack of specificity and sensitivity in the methods available to assess OS in vivo or to the fact that these studies do not generally take into account the confounding effects of other factors, which have been shown to significantly increase OS. $F_2$ isoprostane, of which urinary excretion of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) is an integrated index of its production, is currently considered the most reliable marker of LP in vivo.[6] Moreover, an ideal clinical model should allow evaluation of the effect of relevant changes in glycemic status without the possible artefactual effects of the metabolic consequences of chronic hyperglycemia or the presence of diabetic complications. Thus, the aim of this study was to evaluate the effect of normalization of blood glucose levels on urinary 8-epi-$PGF_{2\alpha}$ excretion at the onset of type 1 diabetes.

## MATERIALS AND METHODS

We studied 14 type 1 diabetic patients recruited from an adult Diabetes Unit at the onset of the disease (50% males; mean age, 24.3 ± 4.9 years) and 14 healthy volunteers (42% males; mean age, 27.4 ± 3.5). The 2 groups were matched with respect to age and body mass index. All patients were nonsmokers and were normotensive (<130/80 mm Hg). Diagnosis of type 1 diabetes was achieved according to the National Diabetes Data Group criteria.[7] Patients were excluded if, at admission, they presented glycemia > 300 mg/dL or ketoacidosis (indicating the immediate need for insulin therapy), pregnancy, intercurrent disease, consumption of multivitamin supplements or aspirin, or were active smokers. The clinical and biochemical characteristics of the diabetic patients and control subjects are summarized in Table 1.

Informed consent was obtained from each participant, and the Ethical Committee of the Hospital Clínic approved the experimental protocol.

### *Study Design*

After the diagnosis of type 1 diabetes, the patients were hospitalized. In all the subjects of both groups, 24-hour urine collection was performed to determine urinary creatinine, albumin, and 8-epi-$PGF_{2\alpha}$ excretion. Thereafter, a blood sample was obtained after overnight fasting to determine glycemia, creatinine, glycosylated hemoglobin ($HbA_{1c}$), total cholesterol, triglycerides, and high-density lipoprotein (HDL)-cholesterol and low-density lipoprotein (LDL)-cholesterol.

The diabetic patients then initiated insulin therapy with multiple-dose insulin regimens, and in these patients all the determinations were repeated 16 weeks after hospital discharge. One week after starting insulin therapy and having achieved clinical stabilization, the glucagon test was performed to evaluate residual $\beta$-cell function (fasting C-peptide levels before and 6 minutes after an intravenous injection of 1 mg glucagon) and antibodies related to type 1 diabetes mellitus (glutamic acid decarboxylase [GAD] and thyrosine phosphatase-like protein [$IA_2$]) were also determined.

Patients were visited every 4 weeks after discharge to adjust insulin therapy to obtain fasting glycemia between 3.8 to 6.6 mmol/L and postprandial glycemia < 8.8 mmol/L to avoid hypoglycemic events and to adapt diet regimens to the needs of each patient. Comparisons between diabetic patients and control subjects were made at week 16 when metabolic control had improved.

### *Measurements of 8-Epi-$PGF_{2\alpha}$*

Urine was collected over a 24-hour period with 3 mL being stored in polypropylene tubes at −70°C. Urine samples (2 mL) were centrifuged and spiked with 1.34 ng of the internal standard 8-epi-prostaglandin F2$\alpha$-d4 (Cayman Chemical, Ann Arbor, MI). The sample was left to equilibrate, and 0.5 mL 5% potassium hydroxide was added. After 30

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**Table 1. Clinical Characteristics of the Study Subjects**

| | Type 1 Diabetes Mellitus | | | |
|---|---|---|---|---|
| | Baseline | Week 16 | Control | *P** |
| No. | 14 | 14 | 14 | |
| Age (yr) | 24.3 ± 4.9 | - | 27.4 ± 3.5 | |
| Sex (M/F) | 7/7 | - | 6/8 | |
| BMI (kg/m$^2$) | 21.6 ± 3.4 | - | 22.1 ± 3.5 | |
| Hyperglycemia/ketosis | 4/8 | - | - | |
| Fasting glucose (mmol/L) | 11.3 ± 3.9 | 6.9 ± 1.8 | 4.8 ± 0.5 | .001 |
| $HbA_{10}$ (%) | 11.5 ± 2.1 | 5.4 ± 0.6 | 4.1 ± 0.3 | .001 |
| Cholesterol (mmol/L) | 4.8 ± 1.1 | 4.7 ± 1.0 | 4.7 ± 0.9 | |
| Triglycerides (mmol/L) | 1.1 ± 0.3 | 0.8 ± 0.3 | 0.6 ± 0.3 | .002 |

Abbreviations: BMI, body mass index; $HbA_{10}$, glycosylated hemoglobin.

*Diabetic patients: basal *v* week 16.

minutes of equilibration, the sample was adjusted to pH 3 with 3 mol/L hydrochloric acid and centrifuged (5 minutes at 600 × *g*). Prostaglandins in the supernatants were absorbed in a 100-mg LMS polymer cartridge (Varian, Harbor City, CA). After washing, the isoprostane was eluted with 1 mL ethanol.

This extract was further purified using an 8-isoprostane-affinity column as indicated by the supplier (Cayman Chemical). The resulting extract was evaporated and redissolved in 100 μL 0.5% ammonium acetate pH, 6.5 (AMAC). Extracts were analyzed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) using a 50 × 1 mm Symmetry column (3.5-μm particles, Waters, Barcelona, Spain) and a Finnigan TSQ700 triple quadrupole MS provided with an electrospray source (Thermoquest, Barcelona, Spain). A 0.02% AMAC/methanol/acetonitrile solvent gradient was used for isoprostane separation. The flow rate was 50 μL/min. The $(M\text{-}H)^-$ ions from the 8-isoprostane F2α (352.7) and its internal standard (356.7) was selected for fragmentation (collision energy 28 eV, collision pressure 2.1 mTorr). The corresponding product ions at m/z 192.5 and 196.5, respectively, were used for quantification. A urine pool from a healthy volunteer was used to prepare the standard samples for the calibration curves and the quality control samples. The intra- and interassay coefficients of variation of the assay at the 100 pg/mL level were 7% and 16%, respectively.

### *Autoantibody Measurements*

GAD antibody (GADAb) measurements were determined by radiobinding assays and were considered positive when above 2 U/mL. The assay for GADAb in the second GADAb proficiency test achieved 100% sensitivity and 100% specificity. $IA_2$Ab titers were measured in a radiobinding assay and considered positive when above 0.8 U/mL. The interassay and intra-assay coefficient of variation of IA2Ab determination were 7% and 5%, respectively.

### *Laboratory Studies*

Total serum cholesterol, HDL-cholesterol, and triglyceride levels were assessed with standard enzymatic spectrophotometric techniques, and LDL-cholesterol was determined using the Friedewald equation. Fasting blood glucose was measured with the glucose oxidase method and $HbA_{1c}$ was measured by high-performance liquid chromatography. C-peptide was determined using a radioimmunoassay (limit of detection 0.033 mmol/L; intra-assay coefficient of variation 2.6%; interassay coefficient of variation 4.4%) and a commercially available kit (Bick Santeg, Dietzenbach, Germany)

### *Statistical Analysis*

8-Epi-$PGF_{2\alpha}$ was expressed as the ratio of urinary creatinine levels (pg/mg creatinine). Data are shown as the mean ± SD. The Mann-Whitney *U* test was used to compare the 2 groups, and in the diabetic group, the difference between baseline and after insulin therapy values was analyzed with the Wilcoxon signed rank test. Correlations were analyzed by the Spearman rank correlation test. A *P* value < .05 was considered statistically significant.

## RESULTS

Fourteen patients who fulfilled inclusion criteria were evaluated. At admission all patients presented hyperglycemia and 10 ketonuria. All patients showed autoantibodies related to type 1 diabetes, 83% of which were anti-$GAD^+$ and 66% were $IA_2^+$. No differences were noted in any clinical variable evaluated between type 1 diabetic patients and control subjects. In relation to control subjects, urinary 8-epi-$PGF_{2\alpha}$ excretion was significantly higher in type 1 diabetics (66.9 ± 28.9 *v* 39.1 ± 13.8 pg/mg creatinine, $P = .004$). Insulin therapy produced a clear improvement in metabolic control as reflected by a marked reduction in $HbA_{1c}$ (from 11.5% (week 0) to 5.4%. (week 16), $P = .0001$) as well as in the lipid profile with a significant decrease in triglycerides (1.0 ± 0.3 at week 0 and 0.8 ± 0.3 mmol/L at week 16, $P < .002$) and an increase in HDL-cholesterol (1.1 ± 0.2 at week 0 and 1.5 ± 0.3 mmol/L at week 16, $P < .01$) and a tendency to decrease LDL-cholesterol (3.0 ± 1.2 at week 0 and 2.4 ± 0.7 mmol/L at week 16, $P = .06$). This amelioration in metabolic control was associated with a statistically significant reduction in 8-epi-$PGF_{2\alpha}$ (from 92.0 ± 41.5 to 66.9 ± 28.9 pg/mg urinary creatinine excretion, $P = .015$) (Fig 1). No correlation was found between 8-epi-$PGF_{2\alpha}$ and $HbA_{1c}$, total cholesterol, triglycerides, HDL-, and LDL- cholesterol.

## DISCUSSION

At present, numerous data support the concept that LP, a marker of OS, is enhanced in diabetes mellitus.[1-5] However, some studies have shown controversial results, probably related to shortcomings in the methods used, because most studies use indirect measurements of LP including susceptibility of LDL to in vitro oxidation or malondialdehyde determination, techniques which are not very specific or accurate.[8] $F_2$-isoprostanes are produced in vivo by cycloxygenase-independent free radi-

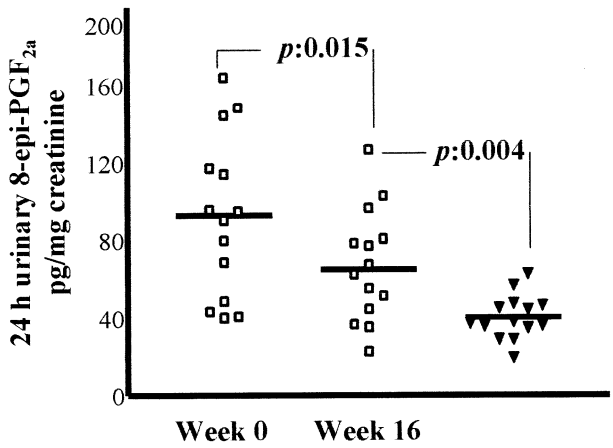


**Fig 1. Urinary 8-epi-$PGF_{2\alpha}$ excretion at onset of clinical type 1 diabetes (□) and in healthy subjects (▼). Symbols represent individual measurements; horizontal bars represent mean value for each group.**

cal peroxidation of arachidonic acid and are released from membrane phospholipids in response to cellular activation, presumably through a phospholipase-mediated mechanism. They circulate in plasma as free forms or as esters in phospholipids, with the free form being excreted in urine.[9] We measured 24-hour urinary excretion of 8-epi-$PGF_{2\alpha}$ not only because it is an integrated index of $F_2$-isoprostane production, but also to avoid potential problems derived from artefactual generation of isoprostanes by autooxidation of arachidonic acid, which may be produced in plasma samples, and to reduce the impact of the previously described day-to-day variation of these parameters.[10,11] We used mass spectometry because of its high specificity and sensitivity.[6] In relation to nondiabetic subjects, in this study we found 40% higher levels of 8-epi-$PGF_{2\alpha}$ in diabetic patients. Our results are in concordance with those recently described by Davi et al[12] who found increased levels of urinary 8-epi-$PGF_{2\alpha}$ in newly diagnosed (3 to 6 weeks) patients with type 1 diabetes mellitus compared with the age- and sex-matched nondiabetic control group.

With indirect measurements of LP, the effect of glycemic control has provided inconsistent results in the literature. Some groups have reported normalization of these levels with glycemic control,[4,5] while in other studies, normal levels were not achieved despite glycemic control.[14,15] The clinical model studied herein, at the onset of type 1 diabetes before and after initiation of insulin therapy, allowed us to analyze the effect of correction of hyperglycemia, without other known factors, which increase LP, such as ageing, metabolic consequences of chronic hyperglycemia, and the presence of diabetic complications. With this model we were able to obtain a very important reduction in blood glucose levels, demonstrated by a difference of $-6$ points in $HbA_{1c}$. This improvement in glycemic control was associated with a significant decrease in urinary 8-epi-$PGF_{2\alpha}$ excretion. Our study cannot rule out the contribution of other metabolic parameters, which also improved with insulin therapy, such as lipid metabolism alterations [especially LDL-cholesterol levels, that has been reported as a determinant of enhanced 8-epi-$PGF_{2\alpha}$ levels,[16] as well as the free fatty acid related to increased ROS generation[17]], ketosis correction,[18] and the effect of insulin per se.[19] We did not find any relationship between 8-epi-$PGF_{2\alpha}$ levels and markers of metabolic control. One can hypothesize that although $HbA_{1c}$ is a good indicator of metabolic control, it reflects only part of the glycemic fluctuations observed in these patients and thus does not replicate the oxidative status. Another finding in this study was that 8-epi-$PGF_{2\alpha}$ levels remained increased in diabetic patients despite achieving stable glycemic control in comparison with control subjects, which could be an early reflection of ongoing improvement in the disease state. Consistent with this argument are the findings of 2 recent studies, which reported that patients with type 1 diabetes melltisu normalize 8-epi- $PGF_{2\alpha}$ levels in long-standing disease ($>$ 1 year) mainly in patients with good glycemic control.[12,13]

In conclusion, in this study we observed that, at the clinical onset of type 1 diabetes mellitus, LP is already increased, and that improvement in glycemic control was associated with a decreased OS early in type 1 diabetes mellitus. Therefore, this is an important argument to recommend the optimization of metabolic control immediately after the diagnosis of type 1 diabetes mellitus to reduce the possible risk of future complications.

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