Association of Type 2 Diabetes With Cyclooxygenase-Mediated Inflammation and Oxidative Stress in an Elderly Population

Johanna Helmersson, MD; Bengt Vessby, MD, PhD; Anders Larsson, MD, PhD; Samar Basu, PhD

Background—Involvement of cyclooxygenase (COX)-mediated inflammation in type 2 diabetes has not been studied, and the association between cytokine-mediated inflammation and diabetes is not fully clarified.

Methods and Results—15-Keto-dihydro-prostaglandin F2α (a metabolite of prostaglandin F2α and an indicator of COX-mediated inflammation), high-sensitivity C-reactive protein (CRP), serum amyloid protein A (SAA), 8-iso-PGF2α (a nonenzymatic, free radical product of arachidonic acid and an indicator of oxidative stress), and α-tocopherol were measured in a population-based sample of 77-year-old men (n=765), in which 112 men had type 2 diabetes. The inflammatory indicators were increased in men with diabetes (urinary 15-keto-dihydro-PGF2α, \(P<0.001\), CRP and SAA, \(P<0.05\)). However, when adjusted for body mass index, waist circumference, or fasting insulin, no association was found between diabetes and CRP or SAA. The oxidative stress indicator 8-iso-PGF2α in urine was increased (\(P<0.01\)) in men with diabetes. Patients who were newly diagnosed with diabetes (<7 years since diagnosis) had increased urinary 15-keto-dihydro-PGF2α and decreased α-tocopherol, but 8-iso-PGF2α was unaltered.

Conclusions—This is the first study to show that type 2 diabetes in elderly men is related to COX-mediated inflammation, reflected by enhanced prostaglandin formation. The high levels of cytokine-mediated acute-phase proteins observed in men with diabetes appear to be related to obesity and increased fasting insulin. The results further suggest that the appearance of chronic inflammation is an early process in the pathogenesis of diabetes, whereas oxidative injury may be a later process, possibly related to inflammation. (Circulation. 2004;109:1729-1734.)

Key Words: diabetes mellitus □ inflammation □ prostaglandins □ free radicals

Type 2 diabetes consists of progressive hyperglycemia, insulin resistance, and pancreatic β-cell failure and is associated with the development of atherosclerosis. Atherosclerosis is considered to be in part a consequence of a chronic low-grade inflammation,1,2 and it has been suggested that atherosclerosis and diabetes might share the same inflammatory basis.3 Inflammatory processes can be studied by measuring cyclooxygenase (COX)-induced or cytokine-mediated products. Prostaglandins and thromboxanes are bioactive compounds derived from arachidonic acid catalyzed by COX and are important mediators of the inflammatory process.4 Prostaglandin F2α (PGF2α) is a major prostaglandin formed in sites of inflammation, and it can be quantified by measurement of 15-keto-dihydro-PGF2α, a major metabolite of PGF2α in plasma. 15-keto-dihydro-PGF2α is shown to be a potent indicator of in vivo COX-mediated inflammatory processes.5-7 Association between diabetes and PGF2α has never been studied, and the role of PGF2α in atherogenesis is yet unknown because of methodological difficulties with the quantification. Cytokine-mediated products (acute-phase proteins) and diabetes have been studied,8,9 but their role in diabetes is not yet clarified.

Oxidative stress is suggested to be associated with macrovascular and microvascular diabetes complications10 and could thus be another possible pathogenetic link between atherosclerosis and diabetes. Prostaglandin-like F2-isoprostanes are formed during free radical–catalyzed, non-enzymatic peroxidation of arachidonic acid and are reliable indicators of oxidative stress in vivo.11 One major F2-isoprostane, 8-iso-PGF2α, is associated with several risk factors for atherosclerosis, including diabetes,12,13 smoking,14 and hypercholesterolemia.15 Depletion of circulating α-tocopherol levels, as a sign of oxidative stress, in diabetic patients has been studied, but results are conflicting: Both unaltered levels and decreased levels have been described.16-18

The aim of the present study was to make a cross-sectional investigation of the association between type 2 diabetes and a marker of COX-mediated inflammation (15-keto-dihydro-PGF2α), cytokine-mediated inflammation (CRP, serum amy-
loid protein A [SAA]), and markers of oxidative stress (8-iso-PGF$_{2\alpha}$, α-tocopherol) in a population-based study. A secondary aim was to study diabetes duration and COX-mediated inflammation and oxidative stress.

**Methods**

**Study Population**
This study is based on the participants from the reinvestigation of the population-based ULSAM cohort (Uppsala Longitudinal Study of Adult Men), which was performed when the participants were 77 years old (mean age, 77.5±0.8 [SD] years). This cohort originally started in 1970, when all men born between 1920 to 1924 and living in Uppsala were invited to participate (2841 men; participation rate, 82%). As previously described.²⁹ In this present reinvestigation, the 1398 men still alive were invited to participate, and 839 were well enough and accepted to participate. The Ethics Committee at Uppsala University approved the study, and all participants gave their informed consent.

**Diabetes Definition and Medication**
Information on smoking habits and medication were obtained by a self-administered questionnaire. Of 839 participants, 765 completed the questionnaire and had a fasting plasma glucose value and thus constitute the present study population finally analyzed. Type 2 diabetes was diagnosed according to the World Health Organization definition²⁹: fasting plasma glucose ≥ 7.0 mmol/L or antidiabetic medication (n = 112). Control subjects had plasma glucose ≤ 7.0 mmol/L and no antidiabetic drugs (n = 650). Men with insulin therapy alone were excluded to avoid the risk of including men with type 1 diabetes (n = 3). Low-dose aspirin treatment was defined as daily intake of 75 to 160 mg acetylsalicylic acid. Information about a history of cardiovascular disease (CVD) (including myocardial infarction, ischemic stroke, or angina pectoris) or heart failure was obtained from the Swedish Hospital Discharge Registry. A subgroup of men without a history of CVD and without intake of low-dose aspirin and nonsteroidal antiinflammatory drugs (NSAIDs) (control subjects, n = 381; diabetes, n = 53) was additionally analyzed.

**Sample Collection and Anthropometric Measurements**
Blood samples were drawn in heparin tubes from the antecubital vein in the morning after an overnight (12-hour) fast. Plasma was separated and stored at −70°C until analysis. Body weight, height, waist circumference, and blood pressure were measured in a standardized way, as previously described.²⁹ Hypertension was defined as a blood pressure >140/90 mm Hg or antihypertensive medication.

**Radioimmunoassays of Urinary 15-Keto-Dihydro-PGF$_{2\alpha}$ and 8-IsopGF$_{2\alpha}$**
Twenty-four-hour urine was collected in 101 men with diabetes and 585 control subjects. The urine samples were immediately frozen at −70°C until analysis. 15-Keto-dihydro-PGF$_{2\alpha}$ was analyzed by radioimmunoassay, with a range of 11.2 to 2867 pmol/0.1 mL. The intra-assay coefficient of variation (CV) was 12.2% at low and 14.0% at high concentrations. Free urinary 8-iso-PGF$_{2\alpha}$ was analyzed by a radioimmunoassay, with a range of 11.2 to 2867 pmol/0.1 mL. The intra-assay CV was 14.5% at low and 12.2% at high concentrations. The cross-reactivity of 8-iso-PGF$_{2\alpha}$ to 15-keto-dihydro-PGF$_{2\alpha}$ was 0.01%. Levels of 15-keto-dihydro-PGF$_{2\alpha}$ and 8-iso-PGF$_{2\alpha}$ were corrected for urinary creatinine values (IL Test; creatinine, 181672-00).

**Assay of High-Sensitivity CRP and SAA**
High-sensitivity CRP and SAA measurements were performed by latex-enhanced reagent (Dade Behring), with the use of a Behring BN ProSpec analyzer (Dade Behring). The intra-assay CV of the CRP method was 1.4% at both 1.23 mg/L and 5.49 mg/L, and the intra-assay CV of the SAA method was 5.9% at 12.8 mg/L and 3.2% at 81.7 mg/L. One CRP outlier value (116 mg/L) and two SAA outlier values (573, 736 mg/L) were excluded from the statistical analysis.

**Biochemical Analyses**
Serum cholesterol, triglycerides, and plasma glucose concentrations were analyzed by enzymatic techniques and HbA1c, with fast performance liquid chromatography (Bio-Rad). Plasma insulin was assayed with an enzymatic immunological assay (Mordacia) in a Coda Automated EIA Analyzer (Bio-Rad Laboratories). Serum α-tocopherol was analyzed by means of high-performance liquid chromatography, with the use of a Hitachi pump and LiChrospher 100 NH2, 250×4 mm column.²² The fluorescence detector had an excitation wavelength of 395 nm and an emission wavelength of 327 nm. Tocopherol levels were corrected for the sum of total cholesterol and triglyceride concentrations. Intra-assay CV for the method was 4.5%.

**Statistical Analyses**
Variables with skewed distribution, according to the Shapiro-Wilks test (W<0.95), were log-transformed to reach normal distribution. Plasma glucose, HbA1c, and SAA did not reach a normal distribution after transformation. Differences between diabetic patients and control subjects were tested by unpaired t test, Mann-Whitney test, or Fisher’s test and linear regression models or partial correlation in multivariate analyses. Associations between continuous variables were tested with Pearson’s or Spearman’s rank correlation analysis. All associations were adjusted for the potential confounders: smoking, body mass index (BMI), hypertension, heart failure and cholesterol (not α-tocopherol and CRP). Associations with CRP and SAA were additionally adjusted for waist circumference and insulin. Probability values <0.05 were regarded as statistically significant. Calculations were performed with Stata 6.0 (Stata Corporation).

**Results**
The characteristics of the study participants are shown in Table 1.

**Levels of 15-Keto-Dihydro-PGF$_{2\alpha}$ (Indicator of COX-Mediated Inflammation)**
Men with diabetes had higher levels of urinary 15-keto-dihydro-PGF$_{2\alpha}$ than did control subjects (Figure 1A). In a subgroup analysis, excluding men with CVD, low-dose aspirin, and NSAID medication, diabetic patients had higher levels than did control subjects (Figure 1B). The association did not substantially change when adjusted for potential confounders (data not shown).

**Levels of CRP and SAA (Indicators of Cytokine-Mediated Inflammation)**
Diabetic patients had higher levels of CRP than did control subjects (Figure 2A). This was also observed in the subgroup without CVD, low-dose aspirin, or NSAID medication (Figure 2B). Adjustment for smoking did not affect the association, but further adjustment with hypertension or heart failure attenuated the association (P=0.09, P=0.07, respectively). Diabetic patients had higher levels of SAA than did control subjects (Figure 2C), which was also observed in the subgroup without CVD, low-dose aspirin, or NSAID medication (Figure 2D). Adjustment for smoking and cholesterol did not affect the association, but further adjustment with hypertension or heart failure attenuated the association (P=0.08, P=0.06, respectively). Adjustment with either BMI, waist circumference, or insulin, however, removed the difference.
Levels of 8-Iso-PGF_2α (Indicator of Oxidative Stress) and α-Tocopherol (Antioxidant)

Diabetic patients had higher levels of urinary 8-iso-PGF_2α than did control subjects in the whole study cohort and in the subgroup without CVD, low-dose aspirin, and NSAID medication (see Figure 3). The association did not substantially change when potential confounders were adjusted for (data not shown). Serum α-tocopherol was significantly lower \((P<0.005)\) in men with diabetes \((1.50±0.26 \text{ mg/mmol}, \text{ mean±SD})\) than in control subjects \((1.57±0.27 \text{ mg/mmol})\). The association was still significant when potential confounders were adjusted for \((P=0.025)\).

Correlation Between Inflammatory Indicators, Antioxidants, and Metabolic Measurements

Correlations of indicators of inflammation and α-tocopherol and metabolic measurements are shown in Table 2. Urinary 15-keto-dihydro-PGF_2α correlated only with 8-iso-PGF_2α. CRP and SAA correlated positively and α-tocopherol correlated negatively with insulin, BMI, and waist circumference. Urinary 8-iso-PGF_2α did not correlate with glucose, HbA_1c, insulin, BMI, or waist circumference (data not shown).

Effects of Disease Duration

Fifty-seven men were diagnosed with diabetes at the earlier reinvestigation at age 70. Prostanoids, α-tocopherol, and diabetes duration, \(<7\) years or \(\geq7\) years, are shown in Table 3. Men at 77 years of age who were diagnosed with diabetes \(\geq7\) years ago had increased levels of both urinary 15-keto-dihydro-PGF_2α and 8-iso-PGF_2α, whereas those who were newly diagnosed with diabetes \((<7\) years ago) had increased 15-keto-dihydro-PGF_2α and decreased α-tocopherol, but 8-iso-PGF_2α was not altered. Cholesterol, triglycerides, insulin, BMI, history of CVD, smoking, statin medication, low-dose aspirin, hypertension, and heart failure did not differ between the two subgroups of diabetic patients.
Discussion

15-Keto-dihydro-PGF$_{2\alpha}$ was clearly related to diabetes in this cross-sectional study of 77-year-old men, independent of cholesterol, smoking, BMI, hypertension, heart failure, previous myocardial infarction, angina pectoris, ischemic stroke, low-dose aspirin, or NSAIDs. Our study is the first to report an increased level of a PGF$_{2\alpha}$-metabolite, which corresponds to the level of the COX-mediated PGF$_{2\alpha}$ in diabetes. Even patients with newly diagnosed diabetes (<7 since diagnosis) had higher levels of 15-keto-dihydro-PGF$_{2\alpha}$ than did control subjects. Prostaglandins are well known mediators of inflammation, and 15-keto-dihydro-PGF$_{2\alpha}$, a metabolite of bioactive PGF$_{2\alpha}$, is a potent indicator of in vivo inflammatory processes. Thus, the results from this study suggest an ongoing COX-related low-grade inflammatory process among patients with type 2 diabetes, both as an early event and a later process in the development of the diabetic disease. PGF$_{2\alpha}$ is shown to be a potent vasoconstrictive compound that might have contributed to the pathology of diabetic complications. Furthermore, it has been shown that a metabolite of thromboxane A$_2$, a COX-mediated product primarily formed in the thrombocytes, is associated with type 2 diabetes.

Elevated levels of CRP are shown to predict diabetes development. However, CRP has not convincingly been cross-sectionally associated with diabetes. A cross-sectional relation between diabetes and increased CRP or other acute-phase proteins has been reported; however, the possible confounding effect of CVD was not investigated. It has been proposed that only diabetic patients with macrovascular disease or syndrome X have higher CRP levels than do healthy control subjects. This present study indicates that CRP and SAA are associated with diabetes independent of previous angina pectoris, myocardial infarction, or ischemic stroke. An earlier study indicated increased levels of SAA in diabetic patients independent of macrovascular disease; however, measures of obesity were not taken into account. CRP and SAA are induced by interleukin-6 (IL-6), which in part derives from adipose tissue. Enhanced IL-6 levels is cross-sectionally related to type 2 diabetes and is shown to predict type 2 diabetes and myocardial infarction. In this present study, there was a positive correlation between CRP or SAA and BMI, waist circumference, and insulin, respectively. Correlations between CRP and BMI have been previously reported. When associations between CRP or SAA and diabetes were adjusted for BMI, waist circumference, or insulin in our study, there was no longer a significant difference between diabetic patients and control subjects. Rather, this would indicate that high CRP and SAA in this study of elderly men are related to obesity and elevated fasting insulin, features often manifest in type 2 diabetes.

Levels of urinary 8-iso-PGF$_{2\alpha}$ were higher and serum α-tocopherol lower in men with diabetes, independent of potential confounders. Elevated levels of 8-iso-PGF$_{2\alpha}$ and lipid hydroperoxides and reduced levels of α-tocoph-
eral\textsuperscript{16,17} among patients with type 2 diabetes have previously been shown. Because 8-iso-PGF\textsubscript{2\alpha} is regarded as the most reliable indicator of oxidative stress in vivo,\textsuperscript{31} the results in this study, together with earlier studies, clearly indicate a higher level of oxidative stress in patients with type 2 diabetes. Studies have suggested that patients with coronary artery disease might have reduced levels of \( \alpha \)-tocopherol,\textsuperscript{17} which gives further support to the oxidative stress hypothesis in atherogenesis.

Those with newly diagnosed diabetes (<7 years ago), however, did not show elevated 8-iso-PGF\textsubscript{2\alpha}, but had reduced levels of \( \alpha \)-tocopherol. The diabetic patients with a disease duration of ≥7 years had elevated levels of 8-iso-PGF\textsubscript{2\alpha} but \( \alpha \)-tocopherol levels comparable to normal. These results seem inconsistent. Although a high \( \alpha \)-tocopherol level would theoretically correspond to a low level of 8-iso-PGF\textsubscript{2\alpha}, this is not the case in this setting, as shown by the weak correlation in Table 2. Furthermore, levels of 8-iso-PGF\textsubscript{2\alpha} cannot be altered by \( \alpha \)-tocopherol supplementation in healthy humans.\textsuperscript{32} \( \alpha \)-Tocopherol levels were not altered in patients with type 2 diabetes, although plasma 8-iso-PGF\textsubscript{2\alpha} was elevated.\textsuperscript{18} This weak connection between 8-iso-PGF\textsubscript{2\alpha} and \( \alpha \)-tocopherol could partly support the discrepancy in the results above. It is possible, however, that in the early stage of diabetes, \( \alpha \)-tocopherol is consumed, but the antioxidative defense is sufficient enough not to cause a state of oxidative stress and elevated 8-iso-PGF\textsubscript{2\alpha}. Later, the amount of free radicals becomes greater, and although the \( \alpha \)-tocopherol levels are upregulated, the antioxidative defense is not sufficient, and 8-iso-PGF\textsubscript{2\alpha} levels thus rise. Thus, our results do not support the hypothesis that oxidative stress is an early event during the disease process of diabetes.\textsuperscript{18} According to these observations, we suggest that oxidative stress might be a later process in the development of diabetes, possibly secondary to inflammation.

This is the largest and first population-based study of the association between diabetes and prostaglandins. It is homogeneous with respect to age and sex but has therefore limited generalizability to other age groups, women, and other ethnic groups. The study population is, for natural reasons, heterogeneous with respect to several diseases, which could yield possible confounding factors other than those we have adjusted for. Because of the cross-sectional design of this study, no conclusions of cause and effect can be made. Longitudinal studies of 15-keto-dihydro-PGF\textsubscript{2\alpha} and 8-iso-PGF\textsubscript{2\alpha}, as predictors of diabetes incidence are planned.

It could be speculated that treatment with acetylsalicylic acid (ASA) and supplementation with n-3 fatty acids might be beneficial in the treatment of type 2 diabetes (irrespective of CVD), as these compounds theoretically inhibit the bio-

### Table 2. Crude Linear Correlations Between Indicators of Inflammation, \( \alpha \)-Tocopherol, and Metabolic Measurements in All Participants

<table>
<thead>
<tr>
<th></th>
<th>15-Keto-dihydro-PGF\textsubscript{2\alpha}</th>
<th>CRP</th>
<th>SAA</th>
<th>( \alpha )-Tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( r )</td>
<td>( P )</td>
<td>( r )</td>
<td>( P )</td>
</tr>
<tr>
<td>15-Keto-dihydro-PGF\textsubscript{2\alpha}</td>
<td>...</td>
<td>...</td>
<td>−0.02</td>
<td>0.690</td>
</tr>
<tr>
<td>CRP</td>
<td>−0.02</td>
<td>0.690</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>SAA</td>
<td>0.05</td>
<td>0.157</td>
<td>0.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8-ISO-PGF\textsubscript{2\alpha}</td>
<td>0.36</td>
<td>&lt;0.001</td>
<td>−0.05</td>
<td>0.223</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>0.07</td>
<td>0.055*</td>
<td>0.02</td>
<td>0.623</td>
</tr>
<tr>
<td>HbA\textsubscript{1c}</td>
<td>0.01</td>
<td>0.764</td>
<td>0.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.07</td>
<td>0.068</td>
<td>0.11</td>
<td>0.003</td>
</tr>
<tr>
<td>BMI</td>
<td>−0.04</td>
<td>0.316</td>
<td>0.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>−0.04</td>
<td>0.920</td>
<td>0.21</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Not correlated when patients with diabetes were excluded.

### Table 3. Prostaglandins, \( \alpha \)-Tocopherol, and Diabetes Duration

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=646)</th>
<th>Diabetes Diagnosis &lt;7 y Ago (n=44)</th>
<th>Diabetes Diagnosis ≥7 y Ago (n=57)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>5.4 (5.1–5.9)</td>
<td>7.4 (7.0–8.4)</td>
<td>8.1 (7.2–9.1)*</td>
</tr>
<tr>
<td>HbA\textsubscript{1c}, %</td>
<td>4.6 (4.4–4.8)</td>
<td>5.6 (5.0–6.2)</td>
<td>5.9 (5.6–6.6)†</td>
</tr>
<tr>
<td>15-Keto-dihydro-PGF\textsubscript{2\alpha}</td>
<td>277 (211–360) (n=585)</td>
<td>301 (237–448)$\dagger$ (n=40)</td>
<td>329 (255–435)$\ddagger$ (n=54)</td>
</tr>
<tr>
<td>8-ISO-PGF\textsubscript{2\alpha}</td>
<td>179 (140–230) (n=585)</td>
<td>197 (147–238) (n=40)</td>
<td>236 (185–293)$\S$ (n=54)</td>
</tr>
<tr>
<td>( \alpha )-Tocopherol, mg/mmol</td>
<td>1.57±0.27</td>
<td>1.46±0.23$\S$</td>
<td>1.53±0.28</td>
</tr>
</tbody>
</table>

Prostaglandins are given in median (interquartile range) (pmol/mmol creatinine). Probability values were generated by linear regression analysis and partial correlation analysis. \( \alpha \)-Tocopherol values are shown as mean±SD.

*\( P<0.06 \), †\( P<0.05 \) vs <7 years.

$\S$$P<0.05$, $\ddagger$$P<0.01$, $\S$$P<0.001$ vs control subjects.

$P$\textsuperscript{adj} adjusted for low-dose aspirin treatment, smoking, cholesterol, BMI, hypertension, and heart failure.

$P$\textsuperscript{adj} additionally adjusted for plasma glucose.
synthesis of PGF$_2\alpha$. ASA has antiinflammatory properties by inhibition of COX directly, and n-3 fatty acids compete with arachidonic acid on the binding site of COX, producing prostaglandins of the 3-series with different biological activity than PGF$_2\alpha$, thereby causing an antiinflammatory effect. However, interventional studies with diabetic patients receiving ASA or n-3 fatty acids are necessary to draw any conclusions about the potentially protective effect of these compounds.

In conclusion, the present study suggests involvement of low-grade COX-mediated inflammation and oxidative stress in type 2 diabetes, independent of various potential confounders and CVD. COX-mediated inflammation might be possible pathogenetic links between diabetes and atherosclerosis. Furthermore, the appearance of chronic inflammation seems to be an early process, whereas oxidative injury might be a later and possibly a secondary process in the progression of type 2 diabetes.

Acknowledgments

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References

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