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Divergence between LDL Oxidative Susceptibility and Urinary F_2-Isoprostanes as Measures of Oxidative Stress in Type 2 Diabetes

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Background: **Oxidative stress is pivotal in atherogenesis. Although the most widely used indirect assay to quantify oxidative stress is LDL oxidative susceptibility, direct assays such as urinary F_2-isoprostanes have shown great promise.**

Methods: **We evaluated the utility of both a direct measure of oxidative stress (urinary F_2-isoprostanes) and an indirect measure of copper-catalyzed, LDL oxidation in a model of increased oxidative stress (diabetes). We also evaluated an enzyme immunoassay (EIA) method for urinary F_2-isoprostanes with a gas chromatography–mass spectrometry method.**

Results: **Excellent intraassay and interassay CVs of <4% were obtained with our EIA method. A good correlation was obtained between the two methods ($r = 0.80$; n = 68) of F_2-isoprostane measurement. An excellent correlation for F_2-isoprostane concentrations was obtained between a timed collection vs 24-h urine ($r = 0.96$; n = 46). Baseline F_2-isoprostane concentrations by EIA were significantly higher in both type 2 diabetics with and without macrovascular complications compared with controls ($P < 0.001$). Supplementation with α-tocopherol led to a significant reduction in F_2-isoprostane concentrations in all diabetic patients compared with baseline values (2.51 ± 1.76 compared with 1.69 ± 1.32 ng/mg creatinine; $P < 0.001$). There were no significant differences in LDL oxidation in both diabetic groups compared with controls. α-Tocopherol supplementation led to significant increases in the lag phase of oxidation as measured by 3 indices in all groups.**

Conclusions: **The measurement of urinary F_2-isoprostanes provides a direct measure of in vivo lipid peroxidation and oxidative stress and appears to be superior to an indirect measure, e.g., LDL oxidative susceptibility, in type 2 diabetes.**

Atherosclerotic vascular disease is the major cause of mortality and morbidity in the US. Data continue to accrue supporting the hypothesis that oxidative stress is pivotal in the genesis of the atherosclerotic lesion *(1, 2)*. There are several direct as well as indirect measures for assaying oxidative stress. Although the most widely used indirect method for measuring oxidative stress is the measurement of LDL oxidative susceptibility *(3)*, direct assays such as measurement of urinary F_2-isoprostanes have shown great promise.

Much evidence implicates oxidative modification of LDL in the pathogenesis of atherosclerosis *(4, 5)*. The diabetic state confers an increased propensity to accelerated atherosclerosis. Factors that may contribute to increased oxidative stress in diabetic patients include antioxidant deficiencies (decreased ascorbate, glutathione, and superoxide dismutase), protein glycation (glucooxidation), and increased production of reactive oxygen species (superoxide, hydrogen peroxide) *(6, 7)*. Other evidence for increased oxidative stress in diabetes includes increased oxidative DNA damage as well as increased titers of autoantibodies to oxidized LDL *(8–10)*. However, data on the oxidizability of LDL in diabetic patients are conflicting *(8, 11–14)*.

Recently, the discovery of F_2-isoprostanes, which are prostaglandin-like compounds produced in vivo by free radical peroxidation of arachidonic acid, has allowed for the direct assessment of in vivo lipid peroxidation in plasma *(15–17)*. Thus, quantification of F_2-isoprostanes may provide a reliable direct measure of oxidative stress in vivo. Increased concentrations of F_2-isoprostanes have been reported in type 2 diabetic patients *(18, 19)*, further underscoring the increased oxidative stress present the

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diabetic state. The major drawback in measurement of F_2-isoprostanes is that the methodology involves gas chromatography–mass spectrometry (GC-MS),[3] which, although sensitive and accurate, is laborious and time-consuming and may not be available in many laboratories. This has prompted development of commercial immunoassay methods for the measurement of F_2-isoprostanes.

To date, no studies have compared a direct and indirect measure of increased oxidative stress; the present study was undertaken to evaluate the utility of these indices of oxidative stress, i.e., F_2-isoprostanes and LDL oxidative susceptibility in a model of increased oxidative stress, type 2 diabetes. We also evaluated an enzyme immunoassay (EIA) method for measurement of urinary F_2-isoprostanes and validated it against the "gold standard" GC-MS method. Furthermore, we assessed the effect of α-tocopherol supplementation on urinary F_2-isoprostanes in these diabetic patients.

Materials and Methods

PATIENTS

This study was approved by the Institutional Review Board. Volunteers (n = 75) were recruited after giving informed consent. They were divided into three groups: type 2 diabetic patients without macrovascular complications [(DM2); n = 25]; type 2 diabetic patients with macrovascular complications (DM2-MV; n = 25); and age- and sex-matched healthy controls (n = 25). Selection criteria for the participants have been described previously *(20)*. All volunteers gave informed consent. Fasting blood was obtained from all the participants at baseline and after 3 months of supplementation with all-*rac* α-tocopherol (1200 IU/day) and after a 2-month washout phase. Urine samples collected after 24 h were stored at −70 °C for F_2-isoprostane analysis by EIA and GC-MS. Plasma samples were stored at −70 °C and analyzed for α-tocopherol by HPLC and for LDL oxidation as described previously *(20)*.

For comparison of F_2-isoprostane concentrations in timed vs 24-h urine collection, 46 volunteers were requested to collect a first morning urine sample and a 24-h collection sample on different days. Samples were frozen at −70 °C until analysis of F_2-isoprostane concentrations by EIA.

LDL ISOLATION AND OXIDATION

Fasting blood (60 mL) anticoagulated with EDTA was obtained for studies of LDL oxidation. LDL (density, 1.019–1.063 kg/L) was isolated by preparative ultracentrifugation from plasma collected in EDTA (1 g/L) as described previously *(21)*. After dialysis against 150 mmol/L NaCl and 1 mmol/L EDTA (pH 7.4), LDL was filtered and protein content was measured as described previously *(21)*. After overnight dialysis against phosphate-buffered saline, pH 7.4, LDL (100 ng/L protein) was oxidized with 5 μmol/L copper at 37 °C for 8 h. The time points were 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 8 h, respectively. At the respective time points, LDL oxidation was arrested with 200 μmol/L EDTA and 40 μmol/L butylated hydroxytoluene followed by refrigeration. We used three assays to quantify LDL oxidation to better appreciate lipid peroxidation and aldehydic modification of apolipoprotein B-100 (apo B-100) *(4, 22)*. We determined the amount of conjugated dienes formed by monitoring the absorbance of the LDL sample at 234 nm at various time points using a phosphate-buffered saline blank *(21)*. We measured the lipid peroxide content of LDL by the ferrous oxide-xylenol orange method *(23)*, and we measured apo B fluorescence of LDL samples after dilution in a spectrofluorometer with excitation set at 360 nm and emission at 430 nm using a 5-nm slit width *(21)*. We computed the lag phase of oxidation using the time course curve *(21)* from the three indices of oxidation.

EIA ANALYSIS OF F_2-ISOPROSTANES

Purification and extraction of urine samples was performed before EIA analysis as reported previously *(24)*. The pH of the urine samples was adjusted to <4.0 with 1.0 mol/L HCl, and a 1-mL aliquot of urine was extracted on a Bakerbond SPE C18 column (JT Baker) that had previously been rinsed with 5 mL of methanol followed by 5 mL of ultrapure water (Cayman Chemical; catalog no. 400000). The columns were then washed with ultrapure water, allowed to dry, and then equilibrated with 5 mL of hexane. After eluting with ethyl acetate and 10 ml/L methanol, sodium acetate was added to the eluate, which was then vortex-mixed and subjected to silica gel chromatography. Elution was carried out with ethyl acetate and methanol (1:1 by volume); eluates were then dried under nitrogen and reconstituted with EIA buffer (1 mL). The extracted samples were diluted 1:5 to 1:8 with EIA buffer and assayed according to manufacturer's protocol for the 8-Isoprostane Enzyme Immunoassay method (Cayman Chemical). The EIA exhibits >1% cross-reactivity with 8-isoprostaglandin F3 α (21%), 8-isoprostaglandin E2 (1.8%), 2,3,dinor 8-isoprostaglandin F2 α (1.7%), and 8-isoprostaglandin E1 (1.6%). We measured urine creatinine on the Olympus (Redondo, CA) by the Jaffe reaction using standard techniques. F_2-Isoprostane concentrations are expressed as ng/mg of creatinine.

GC-MS ANALYSIS OF F_2-ISOPROSTANE

We measured F_2-isoprostanes in urine on frozen samples after thin-layer chromatographic purification by GC negative-ion chemical ionization and electron-ionization MS using a deuterated internal standard in the laboratory of Dr. Raymond F. Burk (Vanderbilt University, Nashville,

[3] Nonstandard abbreviations: GC-MS, gas chromatography–mass spectrometry; EIA, enzyme immunoassay; DM2, type 2 diabetic patients without macrovascular complications; DM2-MV, type 2 diabetic patients with macrovascular complications; and apo, apolipoprotein.

TN) *(25)*. Catalytic hydrogenation and formation and analyses of boronate derivatives were carried out as described previously *(25)*.

STATISTICS

Linear regression analysis was performed using the Microsoft Excel software. Comparison of differences between groups was determined by Wilcoxon signed-rank test with the use of Sigma Stat software. The degree of significance was set at <0.05.

Results

PRECISION

Intraassay (n = 5) and interassay (n = 5) CVs of <4% for three concentrations of F_2-isoprostanes were obtained for the EIA method. The precision and accuracy for the GC-MS method were ± 6% and 96%, respectively *(25)*. The lowest limits of detection with the EIA and GC-MS methods were 4 ng/L and 5 ng/L, respectively.

VALIDATION OF EIA VS GC-MS

The EIA assay was validated for F_2-isoprostanes determination in urine by comparison with the GC-MS method. A good correlation was obtained between the two methods (r = 0.80; n = 68; Fig. 1). The median F_2-isoprostane concentrations for the EIA and GC-MS methods were 1.74 and 1.70 ng/mg of creatinine, respectively.

COMPARISON OF TIMED VS 24-H URINE COLLECTION

We assessed the variation in F_2-isoprostane excretion between the first morning urine collection and 24-h urine collection in 46 volunteers. F_2-Isoprostane concentrations were 0.41–10.6 ng/mg of creatinine. An excellent correlation for F_2-isoprostane concentrations was obtained between the two collection methods (r = 0.96; n = 46, Fig. 2). The mean F_2-isoprostane concentrations in timed vs 24-h collection were 2.26 ± 1.79 and 2.38 ± 2.11 ng/mg of creatinine, respectively.

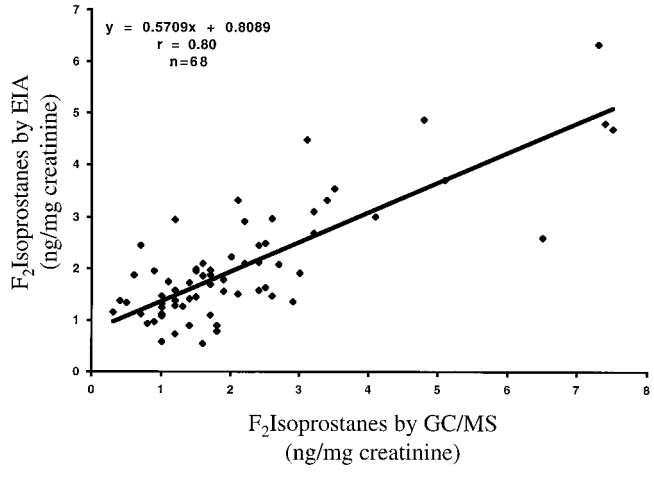

Fig. 1. Validation of enzyme immunoassay for F_2-isoprostanes with GC-MS.

F_2-Isoprostane concentrations were analyzed in urine samples (n = 68) by both the EIA method and by the GC-MS as described in *Materials and Methods*.

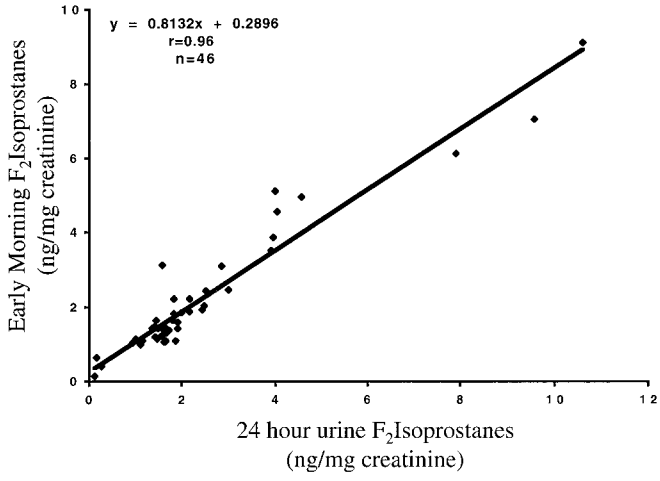

Fig. 2. Comparison of early morning vs 24-h urine for measurement of isoprostanes.

Volunteers (n = 46) were asked to collect an early morning urine sample as well as a 24-h urine collection on different days. Samples were frozen at −70 °C, and F_2-isoprostanes were analyzed in both samples by EIA as described in *Materials and Methods*.

F_2-ISOPROSTANE CONCENTRATIONS IN DIABETIC PATIENTS

F_2-Isoprostane concentrations by EIA were significantly higher in DM2 and DM2-MV patients compared with age- and sex-matched controls (2.03 ± 1.17 and 2.61 ± 1.53 ng/mg creatinine, respectively, compared with 0.71 ± 0.35 ng/mg creatinine; P <0.001, Fig. 3); however, no significant differences existed at baseline between DM2 and DM2-MV for F_2-isoprostane concentrations (P = 0.37). We examined the effect of α-tocopherol supplementation (1200 IU/day) on urinary excretion of F_2-isoprostane in diabetic patients with and without macrovascular complications, using the EIA and GC-MS methods.

α-Tocopherol supplementation was associated with an increase in plasma α-tocopherol concentrations as re-

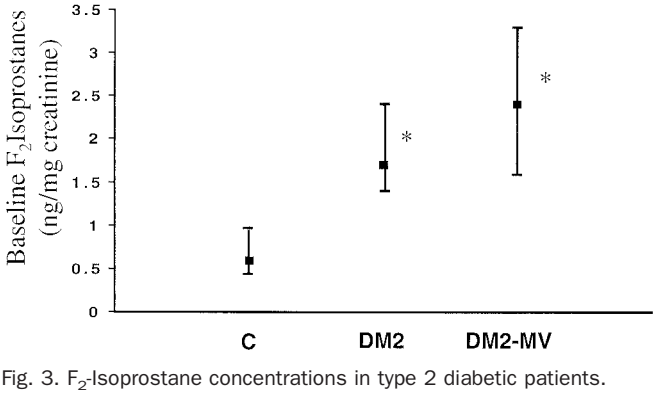

Fig. 3. F_2-Isoprostane concentrations in type 2 diabetic patients.

Twenty-four-hour urine samples were obtained from controls and DM2-MV and DM2 patients and stored at −70 °C. Data represent 25th percentile, median, and 75th percentile of F_2-isoprostane concentrations by EIA in the three groups. *, P <0.001 by Wilcoxon signed-rank test.

Table 1. Effect of α-tocopherol on F_2-isoprostane concentrations in diabetic patients.[a]

	GS-MS		EIA	
	DM2	DM2-MV	DM2	DM2-MV
Baseline	1.7 (1.5, 2.6)	2.5 (1.7, 3.2)	1.7 (1.4, 2.4)	2.4 (1.6, 3.3)
α-Tocopherol phase	1.4 (1.2, 1.6)	1.4[b] (1, 2.1)	1.4 (1.1, 1.6)	1.5[b] (1, 1.7)
Washout phase	1.9[c] (1.6, 2.4)	2.6[c] (1, 3.1)	2.0[c] (1.5, 2.3)	2.6[c] (1.3, 2.9)

[a] Data are presented as median (25th and 75th percentiles).
[b] $P < 0.001$ compared with baseline by Wilcoxon signed-rank test.
[c] $P > 0.6$ compared with baseline by Wilcoxon signed-rank test.

ported previously *(20)*. Supplementation with α-tocopherol led to a significant reduction in F_2-isoprostane concentrations in the combined diabetic group compared with baseline values (2.51 ± 1.76 ng/mg of creatinine compared with 1.69 ± 1.32 ng/mg of creatinine; $P < 0.001$). However, when the diabetic patients were subgrouped into DM2 and DM2-MV, only the reduction in DM2-MV was significant after supplementation ($P < 0.001$; Table 1) in both the EIA and GC-MS methods. In addition, F_2-isoprostane concentrations returned to baseline values after the 6-week washout phase in all groups ($P > 0.6$).

LDL oxidative susceptibility in diabetic patients

LDL oxidative susceptibility was monitored by three indices of oxidation: (*a*) conjugated dienes, (*b*) lipid peroxides, and (*c*) apo B fluorescence. We found no significant differences in lag phase, using all three indices of LDL oxidative susceptibility, in both diabetic groups compared with controls. Furthermore, there was no significant difference between the DM2 and DM2-MV groups (Table 2). α-Tocopherol supplementation led to significant increases in lag phase of oxidation as measured by all three indices in the control, DM2, and DM2-MV groups, respectively, as reported previously for conjugated dienes *(20)*.

Discussion

Cardiovascular disease is the major cause of mortality and morbidity in the US. Oxidative stress plays a crucial role in the genesis and progression of the atherosclerotic lesion. There are several direct as well as indirect measures for assaying oxidative stress. Although the most widely used indirect method for measuring oxidative stress is measurement of LDL oxidative susceptibility, direct assays such as measurement of urinary F_2-isoprostanes have shown great promise. In this report, we have validated an EIA method for quantification of urinary F_2-isoprostanes, sensitive markers of in vivo oxidative stress, and compared the method with the gold standard GC-MS method. In addition, using a model of oxidative stress, type 2 diabetes, we highlighted the divergence between LDL oxidative susceptibility as assessed by three techniques and urinary F_2-isoprostanes.

Table 2. LDL oxidative susceptibility in type 2 diabetic patients and matched controls.[a]

	Controls	DM2	DM2-MV
Conjugated dienes lag phase, min	65.6 ± 11.6	58.6 ± 11.2	56.9 ± 14.9
Lipid peroxides lag phase, min	66.2 ± 8.4	64.5 ± 11.0	63.8 ± 13.9
apo B fluorescence lag phase, min	38.1 ± 18.2	30.1 ± 16.2	29.1 ± 19.2

[a] Data are expressed as mean ± SD.

F_2-Isoprostanes are prostaglandin-like compounds formed in vivo from free radical-catalyzed peroxidation of arachidonic acid, mainly via a noncycloxygenase-dependent mechanism. F_2-Isoprostanes are found in body tissues in the esterified form and in biologic fluids, such as plasma and urine, in the free form *(15–17)*. The relevance of measurement of urinary F_2-isoprostanes with regard to atherosclerosis has been brought forth in many studies *(15–19)*. F_2-Isoprostanes are increased after LDL oxidation by macrophages, endothelial cells, or copper. Increased concentrations have been detected in oxidized LDL and also in patients with established risk factors for premature atherosclerosis, such as diabetes, hypercholesterolemia, and smoking *(15–19)*. F_2-Isoprostanes have been found to localize in foam cells in human atherosclerotic lesions. Furthermore, α-tocopherol supplementation has been found to suppress F_2-isoprostanes and atherogenesis in apo-E-deficient mice. In humans, α-tocopherol supplementation has been shown to lower urinary F_2-isoprostanes in patients with hypercholesterolemia or diabetes *(15–19)*. Also, in a recent report, we showed that α-tocopherol supplementation (400 IU/day) can decrease urinary F_2-isoprostanes in healthy volunteers *(4)*, but this was not confirmed by another recent study *(26)*, probably because of the study's small sample size (n = 5).

F_2-Isoprostanes can be measured accurately and sensitively by a solid-phase extraction procedure, followed by selective-ion monitoring GC-MS, using tritiated prostaglandin F_2-α as internal standard *(25)*. However, although GC-MS methods are the method of choice, they are technique dependent and involve sophisticated instrumentation that is not available in most laboratories. We have shown excellent intra- and interassay precision for the EIA method, as is seen for the GC-MS method. We also demonstrate an excellent correlation with the GC-MS method, and we show that the EIA method can measure accurately on a timed vs 24-h specimen. We show substantial increases in a model of oxidative stress, type 2 diabetes, using this assay and also show that it can be modulated with antioxidant therapy. Proudfoot et al. *(27)* previously compared the measurement of F_2-isoprostanes in urine by EIA and GC-MS and showed a poor correlation; however, only 14 samples were assayed from healthy volunteers, and it is not clear whether both

methods were conducted at the same time. Wang et al. *(28)*, however, measured F2-isoprostanes by both GC-MS and EIA in 9 healthy volunteers and showed a good correlation between the two methods ($r = 0.99$). Thus, the measurement of F_2-isoprostanes by EIA may provide a sensitive, specific, and noninvasive method for the assessment of in vivo lipid peroxidation in humans, a method that is simpler and less expensive. Furthermore, large numbers of samples can be quantified at the same time with this method. Whereas the measurement of urinary F_2-isoprostanes is a direct measure of oxidative stress, LDL oxidative susceptibility is an indirect measure of oxidative stress. LDL is isolated from plasma and then subjected to oxidative stress, whereas F_2-isoprostanes are directly measured in urine *(29)*. Furthermore, in disease states, the patients are on drugs that could potentially partition in LDL and alter its oxidative susceptibility. Thus, isoprostanes appear to be superior in this case. Also, it is possible that we may have seen significant differences in LDL oxidizability with larger sample sizes. Because urinary F_2-isoprostanes could also derive from local production in the kidney, caution should be exercised in using this as a measure of oxidative stress in patients with chronic renal failure *(25)*. It should also be pointed out that the EIA, although largely specific for 8-isoprostane, also exhibits cross-reactivity with certain other prostaglandins. Furthermore, as pointed out by Lawson et al. *(30)*, all of the 64 possible isomers of F_2-isoprostanes share the same ring structure, and it is believed that prostaglandin antigenicity is largely directed toward the ring. Thus, it is possible that the antibody in this EIA also recognizes other isoprostanes, accounting for the similar concentrations obtained with GC-MS.

Although the diabetic state has been shown to have increased oxidant stress, as evidenced by increased concentrations of superoxide release and antioxidant deficiencies as well as increased F_2-isoprostane concentrations by GC-MS, there is conflict in the literature with regard to in vitro susceptibility of LDL to oxidation, as assessed by the lag phase in type 2 diabetic patients *(8, 11–13, 20, 31)*. In our study, LDL oxidative susceptibility, as measured by lag phase using three indices, conjugated dienes, lipid peroxides, and apo B fluorescence, was not significantly increased in type 2 diabetic patients *(20)*. In this report, we also show that α-tocopherol supplementation leads to a decrease in LDL oxidative susceptibility *(20)*. This finding confirms the reports of previous investigators *(32, 33)*.

Thus, measurement of urinary F_2-isoprostanes provides direct measure of lipid peroxidation and whole body oxidative stress and appears to be superior to indirect measures, such as LDL oxidative susceptibility, in certain conditions of increased oxidative stress, such as type 2 diabetes.

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