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Women and Smokers Have Elevated Urinary F_2-Isoprostane Metabolites; a Novel Extraction and LC-MS Methodology

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Abstract

F_2-Isoprostanes (F_2-IsoPs), regio- and stereoisomers of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and urinary F_2-IsoP metabolites including 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (2,3-dinor-8-iso-$PGF_{1\alpha}$ (2,3-dinor-F1)) and 2,3 dinor-8-iso-$PGF_{2\alpha}$ (2,3-dinor-F2), have all been used as biomarkers of oxidative stress. A novel method was developed to measure these biomarkers using a single solid phase extraction (SPE) cartridge, separation by HPLC, and detection by negative mode selected reaction monitoring (SRM) mass spectrometry (MS), using authentic standards of $PGF_{2\alpha}$; 8-iso-$PGF_{2\alpha}$; 2,3-dinor-F1 and 2,3-dinor-F2 to identify specific chromatographic peaks. The method was validated in a population of healthy, college-aged nonsmokers (n = 6M/8F) and smokers (n = 6M/5F). Urinary F_2-IsoP concentrations were ~0.2–1.5 μg/g creatinine, 2,3-dinor-F1 was ~1–3 μg/g and 2,3-dinor-F2 was ~3–5 μg/g. Additional F_2-IsoPs metabolites were identified using SRM. The sum of all urinary F_2-IsoP metabolites was 50–100 μg/g creatinine indicating their greater abundance than F_2-IsoPs. Women had higher F_2-IsoP metabolite concentrations than did men (MANOVA, main effect $p = 0.003$); cigarette smokers had higher concentrations than did nonsmokers (main effect $p = 0.036$). For men or women, respectively, smokers had higher metabolite concentrations than did nonsmokers ($p < 0.05$). Thus, our method simultaneously allows measurement of urinary F_2-IsoPs and their metabolites for the determination of oxidative stress.

Keywords

oxidative stress; LC-MS; F_2-Isoprostanes; F_2-Isoprostane metabolites; prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); cigarette smoking; gender difference; 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$; 2,3 dinor-8-iso-$PGF_{2\alpha}$

INTRODUCTION

F_2-Isoprostanes (F_2-IsoPs, Figure 1) are regio- and stereoisomers of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) formed by the free radical-mediated oxidation of arachidonic acid (1). Thus, F_2-IsoPs, particularly the 15-series regioisomer, 8-iso-$PGF_{2\alpha}$, are often used as biomarkers of oxidative stress (2,3), and have been measured using well-established GC-MS and ELISA methods (4). Recent advances in LC-MS methods have allowed faster and simpler sample preparation (4, 5).

Plasma or tissue F_2-IsoPs concentrations provide a “snap-shot” assessment of oxidative stress. However, concerns have arisen due to the potential for artifacts arising from in vitro lipid oxidation changes during sample preparation (6). Thus, measurement of urinary F_2-IsoPs was

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proposed to be more reliable and less prone to artifactual increases in measured products thereby allowing for the more reliable assessment of oxidative stress in vivo. (6). Urine also contains appreciable levels of F_2-IsoP metabolites (4,6,7), which could be measured simultaneously to bolster urinary F_2-IsoP results. Additionally, urine can be collected non-invasively from humans. Thus, urinary F_2-IsoPs may provide a better assessment of oxidative stress than samples from other biological materials, such as plasma.

Potentially, urinary F_2-IsoP metabolites may be better biomarkers of oxidative stress (4,6,7). Roberts et al (6) determined that 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (2,3-dinor-F1, Figure 1) was the major metabolite of 8-iso-$PGF_{2\alpha}$. Chiabrando et al (4) also identified 2,3-dinor-8-iso-$PGF_{2\alpha}$ (2,3-dinor-F2) as an additional urinary 8-iso-$PGF_{2\alpha}$ metabolite. Interestingly, both 2,3-dinor-F1 and -F2 were present in similar urinary concentrations. Although both groups developed GC-MS procedures for the identification of these compounds (4,8), the GC-MS methodology described by Nourooz-Zadeh et al (7) facilitated the simultaneous measurement of these metabolites along with the parent compound, 8-iso-$PGF_{2\alpha}$. Importantly, there is consensus among these investigators that the F_2-IsoPs metabolites, 2,3-dinor-F1 and -F2, are present in significantly greater urinary concentrations than is 8-iso-$PGF2_{\alpha}$, suggesting that the metabolites may be more easily measured biomarkers. Moreover, F_2-IsoP metabolism may be important in decreasing adverse consequences of oxidative stress and increasing removal of oxidatively damaged lipids (9).

LC-MS methodologies for the determination of these metabolites have been developed (5, 10). These methods stressed high sample throughput by using short HPLC columns (e.g. rocket method) and reported a single value for the F_2-IsoP metabolitess. However, the metabolites from the 64 possible isomers of F_2-IsoPs are likely to be numerous. Moreover, it is unlikely that all species from a biological sample having the same molecular-ion-to-product-ion reaction are derived from a single parent compound. Thus, the evaluation of F_2-IsoP metabolites resolved by prolonged HPLC separation as we described for plasma F_2-IsoPs (11), may be more advantageous than existing rocket methods because quantitation of specific biomarkers could potentially provide information concerning metabolism of specific precursors.

To this end, we developed a method that uses one solid phase extraction (SPE) cartridge, followed by prolonged gradient HPLC-MS-MS, to identify and quantitate urinary F_2-IsoPs and their metabolites, specifically including 2,3-dinor-F1 and -F2. Additionally, we have identified several other potential F_2-IsoP metabolites using selected reaction monitoring (SRM). The procedure was validated by assessing urinary F_2-IsoPs and their metabolites from a cohort of otherwise healthy nonsmokers and smokers, described previously (12). Free F_2-IsoPs were measured previously in the plasma from these individuals (12), as were total (free + esterified) 15-series F_2-IsoPs (11). While mean F_2-IsoP concentrations were numerically higher among smokers compared to nonsmokers, the differences were not statistically significant for any measured analyte due to large within-group variation. Thus, the simultaneous quantitation of urinary F_2-IsoPs and their metabolites from this same cohort will assess the utility of measuring F_2-IsoPs in urine versus plasma as well as measuring F_2-IsoP metabolites versus parent F_2-IsoPs in urine.

EXPERIMENTAL PROCEDURES

Materials

HPLC grade solvents and reagents were obtained from VWR (West Chester, PA). Purified water was obtained from a Millipore Milli-Q apparatus (Billerica, MA). Authentic samples of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), $PGF_{2\alpha}$, PGE_2, 2,3-dinor-8-iso-$PGF_{1\alpha}$, and 2,3-dinor-8-iso-$PGF_{2\alpha}$, and the deuterated internal standards 8-iso-$PGF_{2\alpha}$-d_4 and PGE_2-d_4 were obtained commercially (Cayman Chemical; Ann Arbor, MI).

Study Design and Sample Collection

The study protocol for assessing vitamin E pharmacokinetics and oxidative stress markers in smokers and nonsmokers was reported previously (12). The protocol was approved by the Institutional Review Board for the protection of human subjects at Oregon State University. At the time of the study, participants consented to the use of archived specimens for the continued assessment of oxidative stress biomarkers.

In brief, healthy, 18–35 year old male and female nonsmokers (n = 6M/8F) and smokers (n = 6M/5F; 10–20 cigarettes/d) were recruited from the university area (12). Participants were not nutritional supplement users (>6-mo) and they maintained normal exercise patterns (<7 h/wk aerobic exercise). The study was designed as a randomized, double-blind, placebo-controlled vitamin C intervention study in which participants received vitamin C (500 mg; twice daily) or matching placebo for 17 d. On d 14, participants received an equimolar mixture of deuterium-labeled α- and γ-tocopherols (~50 mg each d_6-α- and d_2-γ-tocopheryl acetates) on a single occasion and blood was collected in regular intervals from 0 to 72 h following labeled-tocopherol administration. Urine was collected in 8 h intervals from 0 to 24 h, aliquoted, and subsequently stored at −20° C. The last 8 h collection (16–24 h) contained the first void on the second day of the trial. At the completion of the trial, all participants underwent a 3-month washout period, and subsequently returned to the study center to complete the alternate arm of the study as described. During each supplementation period, participants were instructed to follow a low ascorbic acid diet; plasma ascorbic acid was reported previously (12).

Plasma unesterified F_2-IsoPs were measured previously using GC-MS (12). Plasma samples were analyzed previously for total (free + esterified) F_2-IsoPs by HPLC-MS-MS (11). The data reported herein from 24 h urine collections are limited to those participants who completed the vitamin C intervention and respective placebo trial and provided complete urine samples from each 8 h collection period. Thus, the present investigation is limited to nonsmokers (n = 6M/8F) and smokers (n = 6M/5F). Smokers had lower body mass indexes (BMI) than nonsmokers and higher cotinine concentrations; women had higher high density lipoprotein (HDL) cholesterol concentrations (Table 1). Otherwise, there were no major differences between genders or smoking status groups.

Sample Preparation

Urine was thawed at room temperature, mixed by inversion, and centrifuged (200 × *g*, 5 min). The supernatant (600 μL) was mixed with 500 μL methanol, internal standards (IS, 12 ng 8-iso-$PGF_{2\alpha}$-d_4 and 3 ng PGE_2-d_4 in 24 μL 1:1 methanol:water) added, followed by addition of buffer (2.0 mL 0.02 M bis-tris-HCl) with mixing at each step. The diluted sample pH was then adjusted using H_3PO_4 or KOH to pH 6.0 ± 0.05.

Strata X-AW cartridges (100 mg/3 mL, Phenomenex, Torrance, CA) were each pre-conditioned with 2 mL 98:2 methanol:formic acid (v:v) and subsequently equilibrated with 2 mL water. Diluted urine samples were loaded, the cartridges washed with 2 mL water, followed by 4 mL 25% methanol, 2 mL acetonitrile, and cartridges dried under a gentle vacuum (~5 mm Hg, 30 sec). Cartridges were then eluted 3-times with 1 mL methanol, and the eluants from each cartridge pooled and collected into glass tubes. Samples were dried under nitrogen gas, reconstituted in 200 μL methanol containing 0.1% formic acid (v:v), and injected onto the LC-MS-MS.

Chromatography and Mass Spectrometry

Separation was carried out using a Shimadzu HPLC system (Columbia, MD) consisting of two LC-10ADvp pumps, a DGU-14A degasser, a SIL-HTc autosampler-system controller maintained at 10° C, and a CTO-10Avp column oven (35° C) with a Synergi Hydro-RP column

(250 × 2 mm i.d., 4 μm) equipped with a SecurityGuard C_{18} guard cartridge (4 × 2 mm; both from Phenomenex; Torrance, CA). Mobile phases A (water) and B (methanol) contained 0.01% acetic acid (v/v) and were delivered at a total flow of 200 μL/min using the following gradient scheme: 5 min equilibration at 30% B prior to injection, 30% B from 0–0.5 min, followed by a linear gradient over 1 min to 58% B, a linear gradient over 13.5 min to 66% B, a linear gradient over 7 min to 69% B, then to 100% B over 2 min which was held until the completion of the run at 32 min.

The HPLC system was coupled to a triple quadrupole mass spectrometer with TurboIon Spray source operated with both mass analyzers set at unit resolution in negative mode (Applied Biosystems/MDS Sciex API 3000, Foster City, CA). Nebulizer, curtain, and collision (CAD) gas parameters were set at 12, 10, and 7, respectively. Heater gas was supplied at 6 L/min at 425°C. All gases were high purity nitrogen supplied by a custom liquid nitrogen system (Polar Cryogenics, Portland, OR). The ionizing voltage was −4000 V, and the declustering, focusing, entrance, and exit potentials were −50, −250, −8, −35, and −11 V, respectively.

To identify chromatographic peaks, product ion spectra of authentic standards of 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, 2,3-dinor-F1, and 2,3-dinor-F2 were acquired at collision energies (CE, see below) that produced a molecular ion intensity of 30–80% of the most abundant fragment ion. Then, each standard was analyzed by gradient HPLC and detected by single reaction monitoring (SRM) experimentation based on the major fragment ions in the corresponding product ion spectrum, thereby creating a multi-trace profile for each compound. Next, a human urine sample was analyzed similarly to identify corresponding chromatographic peaks eliciting identical SRM characteristics to each specific standard.

Analytes were detected and quantified using SRM of seven reactions:

15-series PGFs, *m/z* 353 to 193, CE −35 V

8-iso-$PGF_{2\alpha}$-d_4 internal standard, *m/z* 357 to 197, CE −35 V

PGE_2, *m/z* 351 to 189, CE −27 V; PGE_2-d_4, *m/z* 355 to 193, CE −27 V.

2,3-dinor-F1, *m/z* 327 to 283, CE −28 V

2,3-dinor-F2, *m/z* 325 to 237, CE −22 V

Standard curves were constructed at 8 concentrations for 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, PGE_2 (100–4000 pg/mL), 2,3-dinor-F1 (1250–50,000 pg/mL), and 2,3-dinor-F2 (2500–100,000 pg/mL). 8-iso-$PGF_{2\alpha}$-d_4 was used as the IS for all analytes with the exception that PGE_2-d_4 was used for PGE_2. Quantitation was performed using the Analyst 1.41 software (Applied Biosystems/MDS Sciex). The Analyst software calculated signal to noise (S/N) ratios for 2,3-dinor-F1 (*m/z* 327 to 283) ranging from 6 to 50 (15 – 20 as the mode values), when a representative, non-zero background region of a urine sample chromatogram (at 4 – 6 minutes) was chosen as the reference background.

Accuracy and Precision

Assay accuracy was evaluated by standard addition experiments conducted in urine and methanol. Known quantities of 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, PGE_2, 2,3-dinor-F1, and 2,3-dinor-F2 were spiked, in duplicate, into urine or methanol. Spiked urine was extracted as described above and spiked methanol was analyzed by LC-MS without prior extraction. Intra-day- and between-day precision was determined by analyzing five aliquots of urine on five separate occasions throughout a month.

Statistical Analysis

Data are expressed as mean ± SE. Repeated measures MANOVA was performed using JMP Statistical Discovery Software (SAS Institute, Cary, NC) to evaluate effects attributed to smoking status, gender, and with-in subject vitamin C treatment effects and time effects from urine samples collected in three 8 h intervals over 24 h (0–8, 8–16, and 16–24 h). For the analysis of repeated measures (multiple samples from the same subject), the natural logarithm of the dependent variables (urine concentration at the three time points in each study period, or vitamin C treatment) was used to minimize the correlation between the mean and variance of the data. Results were considered to be statistically significant at $p < 0.05$. Vitamin C had no effect on the oxidative stress parameters studied, as has been observed previously (13), so data from time points were averaged, as indicated. Similarly, when other parameters did not reach statistical significance, the values from each subject were averaged and the statistical analysis was repeated using matched pairs or MANOVA with repeated measures, as appropriate. It should be noted that as the numbers of data points decreased the statistical power decreased.

RESULTS

Precision and Accuracy of the SPE extraction and LC-MS analyses

The intra-day and inter-day precision of the SPE extraction and LC-MS analyses were evaluated from five aliquots of a single urine specimen analyzed over one month on five separate occasions. For the five analytes for which standards are available (8-iso-$PGF_{2\alpha}$; $PGF2_{2\alpha}$; PGE_2; 2,3-dinor-F1 and 2,3-dinor-F2), the intra-day coefficients of variation (CV) for each of the standards ranged from 6 to 9%, while the inter-day CVs for each ranged from 10 to 14%.

Assay accuracy was determined by comparing the ratio of the slopes of the line determined from measured amounts recovered from spiked human urine samples (standard addition) with the slope generated from spiked methanol. Standard addition experiments indicated that the accuracy of the assay was 101% for 8-iso-$PGF_{2\alpha}$, 104% for $PGF_{2\alpha}$, 102% for PGE_2, 59% for 2,3-dinor-F1, and 86% for 2,3-dinor-F2. Because the measured concentrations of 2,3-dinor-F1 and F2 were within the quantitative range of the standard curves, the lower accuracy for these analytes was apparently the result of lower extraction recoveries. Therefore, the extraction protocol was optimized for maximum recovery of 2,3-dinor-F1 and -F2.

The standard addition regression estimates were linear in the range of 0, 20, 50, and 100% of standard mix added (equivalent to a urinary concentration of 8-iso-$PGF_{2\alpha}$, 1000 pg/mL; $PGF_{2\alpha}$ and PGE_2, 2000 pg/mL; 2,3-dinor-F1, 5000 pg/mL, and 2,3-dinor-F2 10,000 pg/mL at 100%). The standard curves used for quantitation were linear from 0 to 4000 pg/mL for 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, and PGE_2; 0 to 40 ng/mL for 2,3-dinor-F1; and 0 to 80 ng/mL for 2,3-dinor-F2.

Identification of Metabolites with SRM patterns similar to 2,3-dinor-F1 and 2,3-dinor-F2

Identification of biomarkers of lipid peroxidation is difficult due to the variety of lipid oxidation products, which potentially may be metabolized to an even greater variety of products. In addition, few authentic standards are available commercially. To circumvent these potential limitations, we used a prolonged HPLC gradient to separate multiple peaks from human urine samples and identified those peaks with product ion spectra matching those of available standards.

F_2-IsoPs in human urine were identified tentatively as 8-iso-15*(R)*-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, and $PGF_{2\alpha}$ because they had retention times identical to those of the standards and they had SRM profiles matching the authentic standards, 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ (data not shown).

Product ion spectra of the 2,3-dinor-F1 and -F2 standards (Figure 2A and Figure 3A, respectively) were similar to those reported previously by others (5, 10). The retention times shown from the HPLC-SRM tracings of the 2,3-dinor-F1 and -F2 standards were 11.7 and 11.9 min, respectively (Figure 2B and Figure 3B, respectively). However, when urine sample extracts were analyzed, additional, sometimes larger, peaks had the same SRM profiles as these standards. Three peaks (identified as 2, 3 and 4 in Figure 2C), had SRM profiles that matched 2,3-dinor-F1 (Figure 2B, identified as 1 in 2C). Similarly, three peaks (identified as 1, 3 and 4 in Figure 3C) had SRM profiles that matched 2,3-dinor-F2 (Figure 3B, identified as 2 in 3C).

Peaks are named according to their metabolite class and retention time (e.g. F1-12 from a 2,3-dinor-F1 SRM match eluting at 12 minutes).

F_2-IsoPs in Urine from Smokers and Nonsmokers

The method described herein allows the simultaneous measurement of both F_2-IsoPs and F_2-IsoP metabolites in a single extraction and LC/MS separation. Urinary F_2-IsoPs (Figure 4), specifically 8-iso-$PGF_{2\alpha}$, 8-iso-15*(R)*-$PGF_{2\alpha}$ and $PGF_{2\alpha}$, were measured in three separate urine specimens collected in 8 h intervals over 24 h from smokers and nonsmokers who completed placebo and vitamin C supplementation trials (12). Thus, six samples were analyzed for each subject. Remarkably, neither the time of day, nor vitamin C supplementation had any effect on the urinary excretion of any of the three F_2-IsoPs measured ($p > 0.05$, MANOVA). The consistency of the methodology described herein is readily apparent in that the urinary concentrations for each of the subjects did not vary widely for any of the three F_2-IsoPs measured (Figure 4).

In contrast to our expectation that cigarette smoking would increase all of the urinary F_2-IsoPs identified, we found that only 8-iso-$PGF_{2\alpha}$ concentrations were significantly increased in smokers ($p = 0.012$, MANOVA, main effect of smoking). However, women compared with men (MANOVA, main effect of gender) excreted significantly greater 8-iso-$PGF_{2\alpha}$ ($p = 0.02$), 8-iso-15*(R)*-$PGF_{2\alpha}$ ($p = 0.04$), and $PGF_{2\alpha}$ ($p = 0.015$) concentrations.

Given the lack of statistical significance in the F_2-IsoP concentrations between time points, or with vitamin C supplementation, the concentrations for each of the six samples were then averaged for each subject (three collections for each of the two supplements) and the statistical analyses repeated. Nonsmoking men had the lowest F_2-IsoP concentrations, while women smokers had the highest concentrations (Table 2). In general, urinary $PGF_{2\alpha}$ concentrations were approximately double those of either 8-iso-$PGF_{2\alpha}$ or 8-iso-15*(R)*-$PGF_{2\alpha}$. Although $PGF_{2\alpha}$ is the largest of the 15-series peaks in our samples, it is likely a mixture of $PGF_{2\alpha}$ and ent-$PGF_{2\alpha}$ that we cannot resolve, based on the findings by Yin et al (14), who used a chiral column and showed that the major portion of $PGF_{2\alpha}$ in human urine is the enantiomer (ent) form of $PGF_{2\alpha}$. Thus, this peak is called $PGF_{2\alpha}$ + ent-$PGF_{2\alpha}$.

2,3-dinor-F1 and 2,3-dinor-F2 in Urine from Smokers and Nonsmokers

The urinary F_2-IsoPs metabolites, 2,3-dinor-F1 and -F2, were measured in the same extract and during the same HPLC analyses as described above for the urinary F_2-IsoPs. Similar to the F_2-IsoPs data, no significant effects of vitamin C supplementation on urinary 2,3-dinor-F1 or -F2 concentrations were observed (data not shown). The consistency of the 2,3-dinor-F1 and 2,3-dinor-F2 measurements is readily apparent in the concentrations (averages for each

time point obtained during the vitamin C and placebo trials) shown for the three time points for each group (Figure 5A and B).

Women excreted more 2,3-dinor-F1 than did men (MANOVA, main effect of gender, p = 0.025); cigarette smokers also excreted more 2,3-dinor-F1 than did nonsmokers (main effect of smoking P=0.039, Figure 5A). When 2,3-dinor-F1 concentrations were averaged over the six time points for each subject, women smokers had significantly higher concentrations ($p < 0.05$, 2.3 ± 0.3 μg/g creatinine) than did nonsmokers (women 1.6 ± 0.3, or men 1.2 ± 0.1), or male smokers (1.5 ± 0.3).

Women also excreted more 2,3-dinor-F2 than did men (MANOVA, main effect of gender p = 0.0012). Similarly, cigarette smokers excreted more 2,3-dinor-F2 than did nonsmokers (main effect of smoking p = 0.013, Figure 5B). However, 2,3-dinor-F2 concentrations varied with time (main effect, p = 0.006). When the 2,3-dinor-F2 data were analyzed separately for men and women, interesting differences were apparent. Women smokers excreted more 2,3-dinor-F2 (4.6 ± 0.5 μg/g creatinine) than did nonsmokers (3.2 ± 0.4 μg/g, p = 0.036), but concentrations did not vary significantly with the time of day. In contrast, cigarette smoking had no significant effect on the 2,3-dinor-F2 excretion in men. However, men excreted greater 2,3-dinor-F2 concentrations (3.0 ± 0.3 μg/g creatinine) during the overnight collection (16 to 24 h) compared with 8 to 16 h (2.3 ± 0.2, p = 0.001). The collections taken during the day 0 to 8 h (2.6 ± 0.3 μg/g creatinine) were also greater than during 8 to 16 h (p = 0.03). These latter data suggest that men may have increased F_2-IsoPs metabolism and excretion overnight into morning.

Additional Urinary F_2-IsoPs Metabolites in Smokers and Nonsmokers

In addition to 2,3-dinor-F1 and -F2, we identified using SRM several other potential urinary F_2-IsoP metabolites (Figure 2 and Figure 3, respectively). Pair-wise correlations between all data (n = 144) showed that F1-12 and F1-13 concentrations were correlated with 2,3-dinor-F1 ($r > 0.9$, $p < 0.0000$); F2-12, F2-13 and F2-15 concentrations were correlated with 2,3-dinor-F2 ($r = 0.7$ to 0.9, $p < 0.0000$); while F1-14 had lower correlation coefficients with concentrations of either 2,3-dinor-F1 or -F2 ($r < 0.4$, Table 3). Of particular interest, F1-14 was not correlated ($p > 0.05$) with 8-iso-15*(R)*-$PGF_{2\alpha}$ suggesting that it has a different precursor than do the other metabolites.

Given that all of the "identified" urinary F_2-IsoPs metabolite concentrations (except F1-14) were correlated, the concentrations calculated for each metabolite were summed for each subject at each time point and then averaged for the six time points within each subject. Women had higher F_2-IsoPs metabolite concentrations than did men (MANOVA, main effect p = 0.0033), while cigarette smokers had higher concentrations than did nonsmokers (main effect p = 0.036, Figure 6). Women smokers had higher metabolite concentrations than did men ($p < 0.05$); nonsmoking men had lower concentrations than did women ($P<0.05$).

Although F1-14 concentrations were not used in the above estimation of F_2-IsoPs metabolites, the SRM characteristics of the peaks were consistent with the F_2-IsoPs metabolite standards, 2,3-dinor-F1 and -F2. F1-14 concentrations (averaged over the six time points for each subject) showed that smokers had higher F1-14 concentrations than did nonsmokers (p = 0.025, Figure 7). Unlike all of the other biomarkers of lipid peroxidation that were evaluated and reported herein, there was no statistically significant effect of gender on F1-14 concentrations even when all six data points per subject (rather than averages) were evaluated.

DISCUSSION

The strategy used in the current study was to identify multiple biomarkers of oxidative stress in human urine. Rather than using a rapid passage through the HPLC column to obtain a single peak containing various F_2-IsoPs or their metabolites, the separation was prolonged to resolve individual peaks of interest. Our hypothesis was that some biomarkers may be more sensitive to oxidative stress than others. Our strategy has been effective in that significant differences in urinary F_2-IsoPs were detected between women and men, nonsmokers and smokers (~10 cigarettes/day); differences that our plasma F_2-IsoP assay was unable to detect in these same subjects (11). Perhaps more importantly, we have identified a series of metabolites that have mass spectral characteristics of the relatively low abundance 2,3-dinor-F1 and -F2, but when summed together, these apparent metabolites are excreted in quantities 50 to 100 times greater than the two metabolites for which we have commercially available standards. The urinary F_2-IsoPs were found at concentrations of about 0.2 to 1.5 μg/g creatinine (Figure 4), while urinary 2,3-dinor-F1 was about 1 to 3 μg/g and 2,3-dinor-F2 was about 3 to 5 μg/g (Figure 5). In contrast, the sum of the various F_2-IsoP metabolites was in the range of 50 to 100 μg/g (Figure 6). Thus, these urinary F_2-IsoPs metabolite concentrations are supportive of rapid F_2-IsoP metabolism (15) and the excretion of the metabolites as potentially important in ameliorating the adverse effects of oxidative stress.

To identify F_2-IsoPs and F_2-IsoPs metabolites, we first acquired product ion spectra of the individual standards. We then used these results to design an SRM program for each class of analyte. For example, authentic 2,3-dinor-F1 had a molecular ion of *m/z* 327 (negative mode), and major products ions, in order of abundance, of *m/z* 283, 265, 209, 183, 193, and 221 (Figure 2). The SRM program detected these ions as *m/z* 327 products during an HPLC separation of authentic 2,3-dinor-F1 and also during analysis of a human urine extract. The peaks in the urine extract chromatogram that had SRM profiles resembling those of authentic 2,3-dinor-F1, were likely to have a similar chemical structure, but perhaps with stereochemical differences. Once specific peaks were identified based on SRM profiles as likely members of a particular analyte type, we integrated only those peaks. Note that that Figure 2C and Figure 3C also show peaks in the urine extract that did not have the appropriate SRM profile, thus, were not included in our analyses.

Previous methods have utilized mass spectrometry to identify and quantify components with the same single molecular ion-to-product ion reaction as that of available standards with minimal HPLC separation. We found that human urine chromatograms contained a peak, F1-14, with a product ion spectrum similar to that of 2,3-dinor-F1; however, the amounts were not correlated with F_2-IsoP concentrations (Table 3). These findings suggest that peak F1-14 is not derived from the F_2-IsoPs that we quantitated. Moreover, analyses based solely on tandem MS detection with minimal or no HPLC separation may not provide accurate results. Importantly, unlike every other F_2-IsoP and metabolite we detected, there was no gender effect, just increased F1-14 concentrations in smokers (Figure 7). Without the prolonged HPLC separation we used, this very interesting biomarker would have escaped discovery. F1-14 likely has a molecular structure similar to that of 2,3-dinor-F1, but with perhaps different stereochemistry. Clearly, further investigation is needed to characterize this molecule(s).

The SPE method described is a new innovation compared with previously published methods by others. We sought to extend our LC-MS-MS assay for plasma 15-series F_2-IsoPs (11) for use with urine samples and to simultaneously measure urinary F_2-IsoPs metabolites. However, our initial efforts using liquid-liquid extraction or published SPE protocols (5,10) yielded poor recoveries (~25–50% of spiked internal standard) from urine samples. A generic anion exchange SPE protocol (including a methanol wash, then elution with acidified methanol) using mixed-mode weak anion exchange-reverse phase cartridges gave satisfactory recovery

of the internal standard, 8-iso-$PGF_{2\alpha}$-d_4, when added to water, but not when added to urine. Moreover, when the internal standard was added to an extracted urine sample, only half the expected increase in peak area was observed, suggesting a matrix effect, possibly ion suppression in the mass spectrometer. In the course of developing a new F_2-IsoP extraction method, we observed that when urine samples were loaded on mixed-mode reverse phase-weak anion exchange cartridges at ~1 pH unit above the typical carboxylic acid pK_a (e. g., n-butyric, pH 4.81), the protic solvent, methanol (but not acetonitrile) was capable of eluting F_2-IsoPs and F_2-IsoP metabolites, indicating ionic forces still contributed to F_2-IsoP binding to the cartridge at the lower pH. Acetonitrile could then be used to wash any neutral, nonpolar compounds from the cartridge, as well as any residual water. Good recoveries of F_2-IsoPs and F_2-IsoP metabolites were achieved with neutral methanol elution, while agents that caused ion suppression in the MS were eluted with acidified solvents. These findings explain the low recoveries of urinary F_2-IsoPs and F_2-IsoP metabolites when we used conventional anion exchange methods, in which samples are usually loaded at greater than or equal to 2 pH units higher than the pK_a of the analyte of interest, which in turn, is eluted with acidified solvents.

Intra- and inter-day precision of the final assay compare satisfactorily with previously published methods, and the accuracy of measurements of the F_2-IsoPs was nearly 100%. However, the accuracies of 2,3-dinor-F1 and -F2 were lower. We believe the lower accuracy is due to lower rates of recovery of these analytes from urine. The SPE procedure was more robust for variations in the loading pH and elution volumes when determining recoveries of 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, and PGE_2 than for 2,3-dinor-F1 and -F2. As a consequence, attention was focused on achieving as good recovery as possible of the latter without compromising the recovery of the F_2-IsoPs. Our accuracy of 59% for 2,3-dinor-F1 and 86% for 2,3-dinor-F2 is not as high as the 80% reported for the former (5) or the 98 – 99% reported for the latter (10). Nevertheless, the recoveries were reproducible and were linear over a large range, allowing the use of our protocol in analyzing a set of samples collected from college-age subjects, some of them light smokers and others nonsmokers. Bohnstedt et al. (16) previously reported that reverse phase SPE was unsatisfactory for urine sample preparation; they then developed a liquid-liquid extraction method for F_2-IsoPs. Herein we report a SPE procedure using a different stationary phase chemistry that was satisfactory for both F_2-IsoPs and their metabolites. Bohnstadt et al. (16) reported urinary concentrations of iPF2a from control subjects (216 pg/mg creatinine) that were similar to those we observed of 8-iso-PGF2a (the same compound) from nonsmoking male subjects (280 pg/mg creatinine), while we report higher concentrations in female smokers (530 pg/mg creatinine). Welsh et al. (17) also report satisfactory recoveries of F_2-IsoPs and their metabolites using SPE technology.

In the present study, we observed that smokers had greater urinary 8-iso-$PGF_{2\alpha}$ concentrations than did nonsmokers, but cigarette smoking did not significantly increase urinary 8-iso-15 *(R)*-$PGF_{2\alpha}$ or $PGF_{2\alpha}$ concentrations. We previously reported plasma F_2-IsoPs measured from a similar cohort of otherwise healthy college-aged nonsmokers and smokers. Utilizing GC-MS (12) or LC-MS (11) approaches, smokers had numerically higher plasma F_2-IsoPs but without statistical significance between the groups. In a separate study, plasma F_2-IsoPs were significantly higher among smokers, but their smoking history and frequency (>30 cigarettes/d) were substantially greater than observed in the participants from the present study (18,19). Also, proposed biomarkers of oxidative stress are often tested with a strong stress such as CCl_4 poisoning (20). Thus, significantly greater urinary 8-iso-$PGF_{2\alpha}$ in smokers supports the well-established literature indicating that smoking enhances oxidative stress (21,22) and supports the hypothesis of the present work that specific F_2-IsoPs may be more sensitive biomarkers of oxidative stress in vivo. The latter is important for studies conducted in populations that are otherwise healthy, young, and with a limited history (<5 years) and frequency (~10 cigarettes/d) of smoking, that is, subjects with a relatively low magnitude of

oxidative stress, where decreased variability in the methodology would allow increased sensitivity to detect differences between subjects.

It should be noted that our method also allows quantitation of PGE_2. In general, PGE_2 is formed enzymatically (23), but there is evidence that non-enzymatic generation can also occur (24). Given our interest in oxidative stress markers, and the non-specific origin of PGE_2, this molecule was not quantitated for smokers and non-smokers.

Of particular interest is that plasma F_2-IsoP turnover is rapid (15); therefore, blood levels may vary widely over time with transient oxidative stress. Thus, urinary F_2-IsoPs and their metabolite concentration would be expected to provide a more reliable index of systemic oxidative stress with less variability than that of circulating concentrations. Indeed, our measurements confirmed this notion (Table 2) and demonstrated the significance of measuring these biomarkers in urine.

Consistent with the notion of enhanced oxidative stress in smokers, we observed significantly higher urinary 2,3-dinor-F1 and -F2 concentrations in smokers compared with nonsmokers (Figure 5), as were urinary total F_2-IsoP metabolites (Figure 6). However, to our surprise, we observed that women compared with men had higher urinary concentrations of all F_2-IsoPs measured (Table 2 and Figure 4), as well as urinary F_2-IsoP metabolites (Figure 5–Figure 6) despite men and women having similar characteristics (Table 1). Gender effects on lipid peroxidation or oxidative stress biomarkers have been addressed in clinical studies only to a limited extent and the results have been equivocal. Men had higher urinary F_2-IsoPs and plasma thiobarbituric acid-reactive substances (TBARS) than did women (25), as well as higher plasma malondialdehyde (MDA) (26). However, in a study with nearly 300 men and women, Block et al (27) demonstrated that women had significantly higher plasma MDA and F_2-IsoPs than did men. Moreover, the gender effect was maintained despite efforts to adjust for BMI which would potentially control for differences in adiposity. In the present study, F_2-IsoPs and F_2-IsoP metabolite concentrations were not correlated with any parameter shown in Table 1, including BMI (data not shown). Coudray et al (28) also observed significantly greater evidence of lipid peroxidation in women compared with men in a cohort of nearly 1400 participants. Clearly, additional study is needed to further characterize gender differences on oxidative stress as well as to potentially unravel the mechanism leading to such effect.

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LIST OF ABBREVIATIONS

CE, collision energies; F_2-IsoPs, F_2-Isoprostanes; GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; SPE, solid phase extraction; SRM, selected reaction monitoring; 2,3-dinor-F1, 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$, or 2,3-dinor-8-iso-$PGF_{1\alpha}$; 2,3-dinor-F2, 2,3-dinor-8-iso-$PGF_{2\alpha}$.

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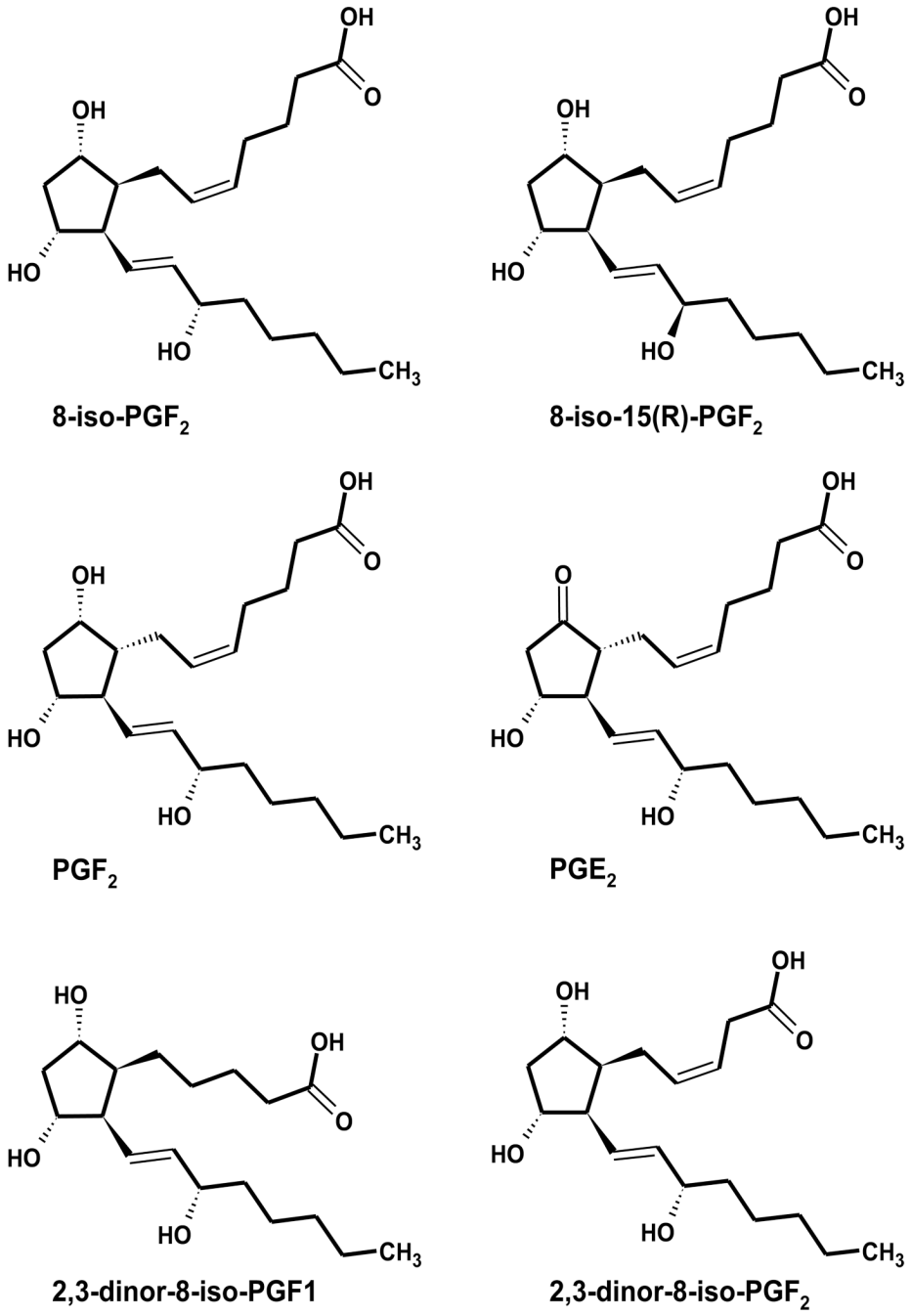

Figure 1. Structures of F_2-Isoprostanes (F_2-isoPs) and metabolites

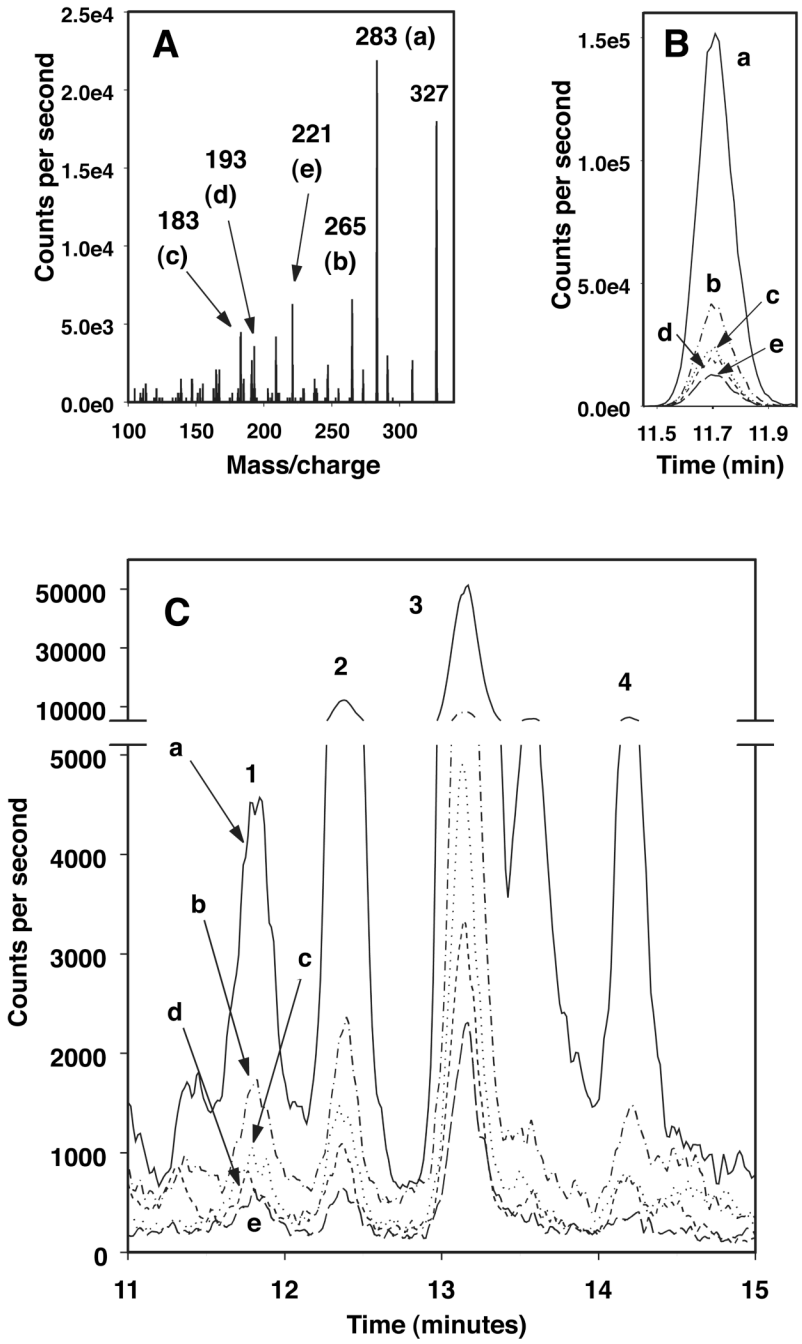

Figure 2. LC-MS identification of 2,3-dinor-PGF$_{1\alpha}$

(A) Fragmentation pattern of 2,3-dinor-PGF$_{1\alpha}$ standard, *m/z* 327 to 283 (a), 265 (b), 183 (c), 193 (d) or 221 (e).

(B) LC-MS chromatogram of 2,3-dinor-PGF$_{1\alpha}$ standard showing tracings of each of the fragments (a–e) identified in A.

(C) LC-MS chromatogram of a urine extract with tracings of peaks identified with SRM profiles showing each of the fragments (a–e, identified in A) that matched those of 2,3-dinor-PGF$_{1\alpha}$ (2,3-dinor-F1, retention time same as the standard, labeled 1; F1-12, labeled 2; F1-13, labeled 3, and F1-14 labeled 4).

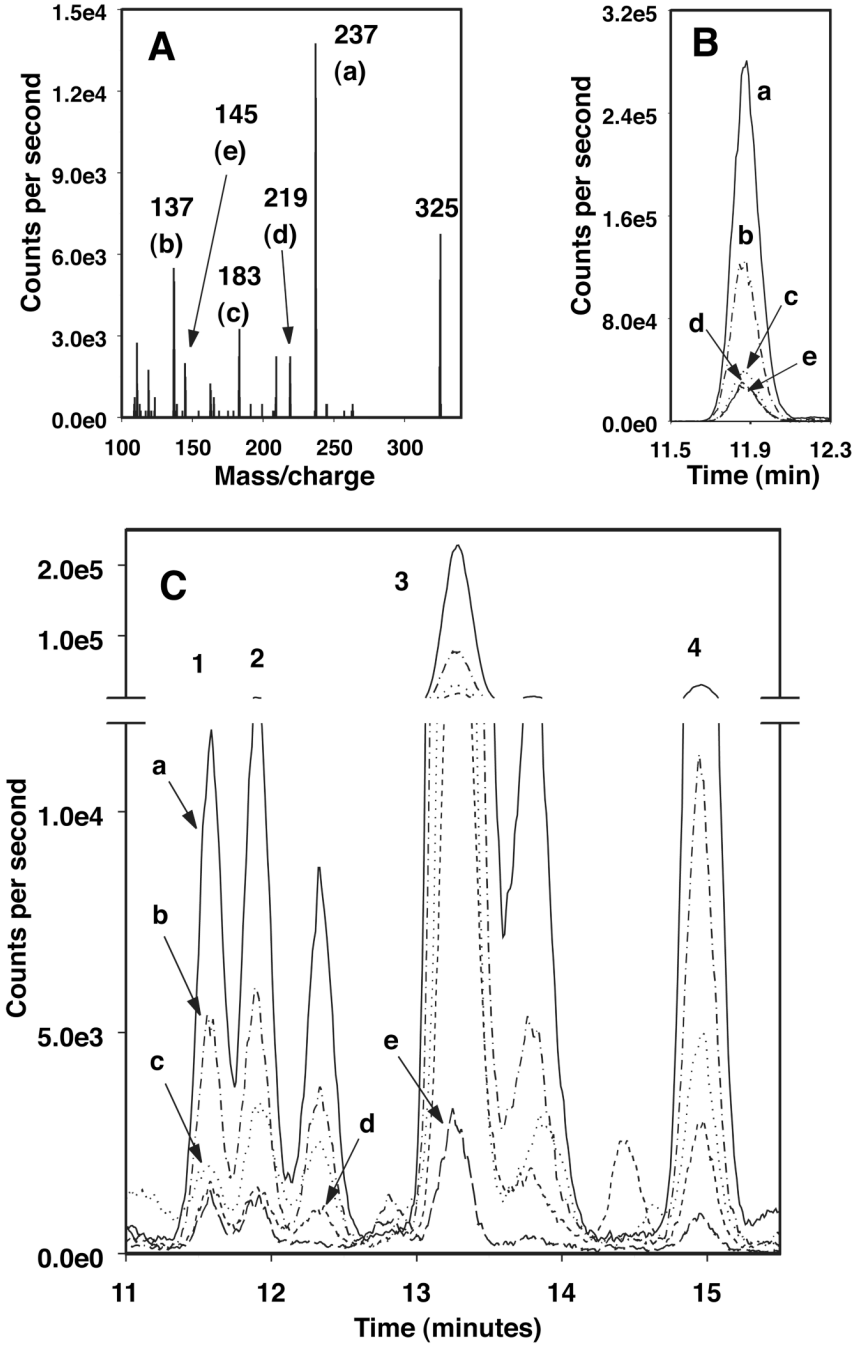

Figure 3. LC-MS identification of 2,3-dinor-PGF$_{2\alpha}$

(A) Fragmentation pattern of 2,3-dinor-PGF$_{2\alpha}$ standard, *m/z* 325 to 237 (a), 137 (b), 183 (c), 219 (d) or 145 (e).

(B) LC-MS chromatogram of 2,3-dinor-PGF$_{2\alpha}$ standard showing tracings of each of the fragments (a–e) identified in A.

(C) LC-MS chromatogram of a urine extract with tracings of peaks identified with SRM profiles showing each of the fragments (a–e, identified in A) that matched those of 2,3-dinor-PGF$_{2\alpha}$ (2,3-dinor-F2, retention time same as the standard, labeled 2; F2-12, labeled 1; F2-13, labeled 3, and F2-15 labeled 4).

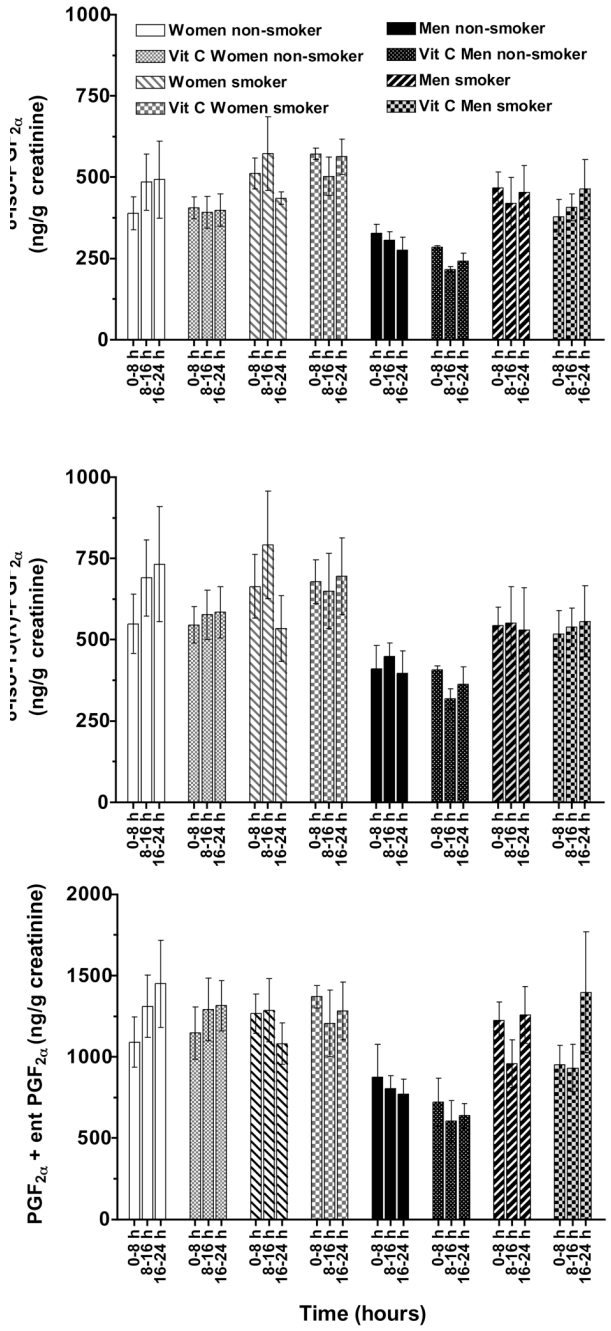

Figure 4. Urinary F_2-isoPs determined over 24 h in smokers and nonsmokers

The urinary F_2-isoPs: 8-iso-$PGF_{2\alpha}$, 8-iso-15*(R)*-$PGF_{2\alpha}$ and $PGF_{2\alpha}$, were quantified both during placebo and vitamin C supplementation in smokers and nonsmokers from 3 separate urine collections (0 to 8 h, 8 to 16 h, and 16 to 24 h after taking deuterated α-tocopherol during a vitamin E kinetics study [12]). Neither the time of day, nor the supplementation with vitamin C had any effect on the urinary excretion of each of the three F_2-isoPs measured ($p > 0.05$, MANOVA). 8-iso-$PGF_{2\alpha}$ concentrations were significantly increased in smokers ($p = 0.012$, MANOVA, main effect of smoking). Women compared with men excreted significantly (MANOVA, main effect of gender) higher concentrations of 8-iso-$PGF_{2\alpha}$ ($p = 0.02$), 8-iso-15 *(R)*-$PGF_{2\alpha}$ ($p = 0.04$), and $PGF_{2\alpha}$+ ent-$PGF_{2\alpha}$ ($p = 0.015$).

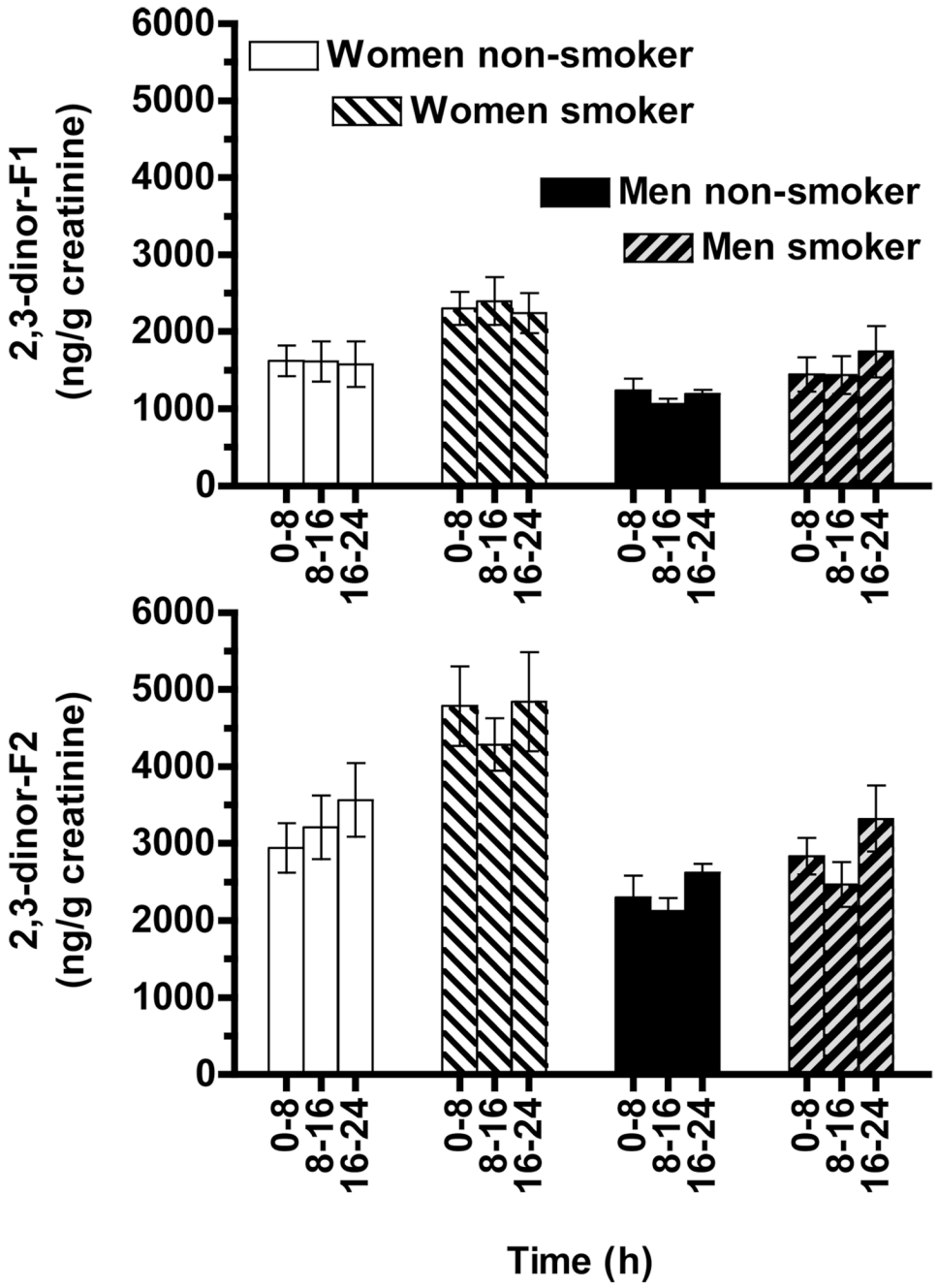

Figure 5. Urinary isoprostane metabolites excreted by smokers and nonsmokers

Urinary 2,3 dinor F1 (A) and 2,3 dinor F2 (B) concentrations, respectively, were averaged for the vitamin C and placebo supplementation trials at each of the three time points.

A. Women excreted higher 2,3-dinor-F1 concentrations than did men (MANOVA, main effect of gender $p = 0.025$); cigarette smokers excreted higher concentrations than did non-smokers (main effect of smoking $p = 0.039$).

B. Women excreted more 2,3-dinor-F2 than did men (MANOVA, main effect of gender $p = 0.0012$); cigarette smokers excreted higher concentrations than did non-smokers (main effects of smoking $p = 0.013$). 2,3-dinor-F2 concentrations also varied with time (main effect, $p = 0.006$).

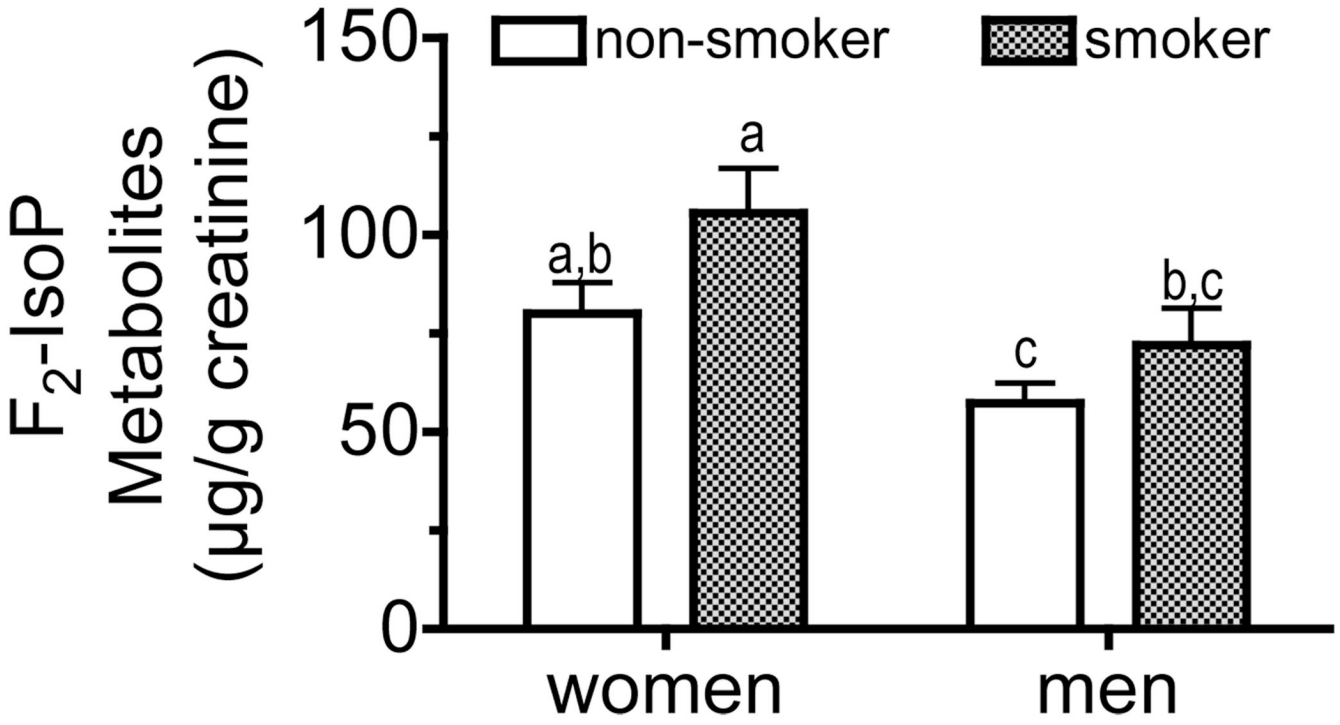

Figure 6. Urinary total isoprostane metabolites excreted by men and women smokers and nonsmokers

Urinary F_2-isoP metabolite concentrations (identified as shown in Figure 2 & Figure 3, except F1-14) were individually calculated, summed for each subject at each time point and then averaged for the six time points for each subject. Women had higher F_2-isoP metabolite concentrations than did men (MANOVA, main effect $p = 0.0033$), while cigarette smokers had higher concentrations than did nonsmokers (main effect $p = 0.036$). Bars not sharing the same letter are significantly different by Tukey's pair-wise comparisons ($p < 0.05$).

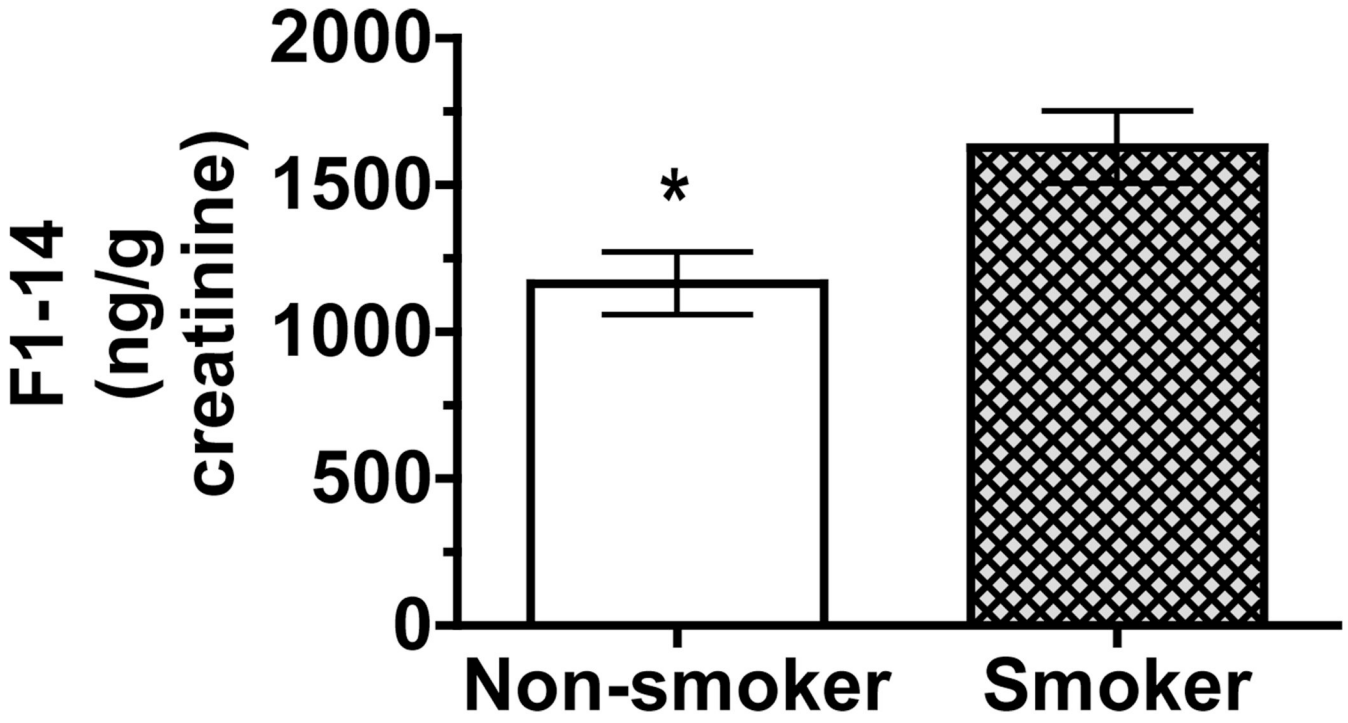

Figure 7. Urinary F1-14 excreted by smokers and nonsmokers

F1-14 (identified in Figure 2C) concentrations were averaged over the six time points for each subject. Smokers had higher F1-14 concentrations than did nonsmokers ($p = 0.025$). There were no significant effects of gender on F1-14 concentrations.

Table 1

Participant Characteristics[1]

Gender	Group	Age (y)	BMI* (kg/m^2)	Serum Lipids: TG (mg/dL)	Serum Lipids: Chole-sterol (mg/dL)	Serum Lipids: HDL-Chole-sterol (mg/dL)	Cotinine (ng/mL)	Cigarettes/d
Women	Non-smoker	22.4 ± 0.7	24.3 ± 1.0^{a}	71 ± 12	164 ± 4	76 ± 5^{a}	28 ± 5^{b}	0^{c}
	Smoker	20.2 ± 0.7	23.4 ± 2.2^{a,b}	92 ± 18	161 ± 13	72 ± 15^{a,b}	1473± 263^{a}	8 ± 1^{b}
Men	Non-smoker	23.0 ± 0.7	26.6 ± 0.9^{a}	81 ± 17	160 ± 18	48 ± 5^{b}	23 ± 5^{b}	0^{c}
	Smoker	22.5 ± 1.3	20.9 ± 0.4^{b}	84 ± 18	155 ± 9	64 ± 5^{a,b}	3037 ± 708^{a}	14 ± 2^{a}
Statistical Summary (MANOVA)								
Gender		NS	NS	NS	NS	0.03	NS	0.022
Smoking		NS	0.014	NS	NS	NS	<0.0001	<0.0001
Inter-action		NS	NS	NS	NS	NS	NS	0.022

*BMI = body mass index (body weight (kg)/height (m^2)

[1]Participant characteristics (mean ± SE) were analyzed using MANOVA and Tukey's post hoc where appropriate for pair-wise comparisons. Main effects are listed in the table and pair-wise comparisons not sharing the same superscript are significantly different ($p < 0.05$).

Table 2

Urinary F_2-Isoprostanes in smokers and nonsmokers[1]

Gender	Group		8-iso-$PGF_{2\alpha}$ (µg/g creatinine)	8-iso-15*(R)*-$PGF_{2\alpha}$ (µg/g creatinine)	$PGF_{2\alpha}$+ ent-$PGF_{2\alpha}$ (µg/g creatinine)
Women	Nonsmoker		0.43 ± 0.06^{a}	$0.61 \pm 0.09^{a,b}$	1.27 ± 0.16^{a}
	Smoker		0.52 ± 0.03^{a}	0.65 ± 0.09^{a}	1.24 ± 0.08^{a}
Men	Nonsmoker		0.28 ± 0.01^{b}	0.39 ± 0.03^{b}	0.74 ± 0.09^{b}
	Smoker		0.43 ± 0.06^{a}	$0.54 \pm 0.09^{a,b}$	1.12 ± 0.15^{a}
Statistical Summary (MANOVA)					
Gender		p =	0.019	0.04	0.015
Smoking		p =	0.012	NS	NS
Interaction		p =	NS	NS	NS

[1] No significant differences were observed for vitamin C supplementation or for each 8 h collection interval; therefore, urinary F_2-IsoPs were averaged within each participant over time and vitamin C supplementation. Urinary F_2-IsoPs were analyzed using MANOVA and Tukey's post hoc where appropriate for pair-wise comparisons. Main effects are listed in the table and pair-wise comparisons within a column not sharing the same superscript are significantly different ($p < 0.05$).

Table 3

Pair-wise Correlations between F_2-IsoPs and their Putative Metabolites

	8-iso-$PGF_{2\alpha}$	**8-iso-15*(R)*-$PGF_{2\alpha}$**	**$PGF_{2\alpha}$+ ent-$PGF_{2\alpha}$**	**2,3-dinor-F1**	**F1-12**	**F1-13**	**F1-14**	**2,3-dinor-F2**	**F2-12**	**F2-13**	**F2-15**
8-iso-$PGF_{2\alpha}$	1.0000	0.9066	0.7938	0.6008	0.5641	0.5797	0.2407	0.6317	0.6360	0.5143	0.4448
8-iso-15*(R)*-$PGF_{2\alpha}$		1.0000	0.7263	0.4304	0.3834	0.4474	0.1090	0.4629	0.4678	0.4286	0.3674
$PGF_{2\alpha}$+ ent-$PGF_{2\alpha}$			1.0000	0.3280	0.3307	0.3458	0.3046	0.4434	0.4372	0.4294	0.4987
2,3-dinor-F1				1.0000	0.9292	0.9399	0.4010	0.8555	0.8552	0.6641	0.4483
F1-12					1.0000	0.9367	0.5028	0.8336	0.8192	0.6879	0.4841
F1-13						1.0000	0.3794	0.8628	0.8729	0.7539	0.4982
F1-14							1.0000	0.2894	0.2636	0.2343	0.3642
2,3-dinor-F2								1.0000	0.9700	0.8790	0.7298
F2-12									1.0000	0.8763	0.7129
F2-13										1.0000	0.8767
F2-15											1.0000

All pair-wise correlations are statistically significant ($P < 0.0001$) with the exception of the correlation of F1-14 with 8-iso-$PGF_{2\alpha}$ ($P = 0.0037$), 8-iso-15*(R)*-$PGF_{2\alpha}$ (not significant), $PGF_{2\alpha}$+ ent-$PGF_{2\alpha}$ ($P = 0.0002$), 2,3-dinor-F2 ($P = 0.0004$), F2-12 ($P = 0.0014$), and F2-13 ($P = 0.0047$).

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