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Original Contribution

URINARY EXCRETION OF THREE NUCLEIC ACID OXIDATION ADDUCTS AND ISOPROSTANE $F_2\alpha$ MEASURED BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY IN SMOKERS, EX-SMOKERS, AND NONSMOKERS

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Abstract—To assess novel liquid chromatography/mass spectrometric methods for measuring oxidative damage to nucleic acids and lipids, we compared urinary excretion of 8-hydroxy-2′-deoxyguanosine (8-OHdG), 5-hydroxymethyl-2′-deoxyuridine (5-OHmU), and 8-hydroxyguanosine (8-OxoG), and an isoprostane, 8-iso-prostaglandin $F_2\alpha$ (Isop$F_2\alpha$) in 234 healthy men ($n = 113$) and women ($n = 121$), 80 current smokers, 96 never-smokers), and 58 ex-smokers (no tobacco use for 3 years). The 8-OHdG and 8-OxoG did not differ significantly by group; 5-OHmU was higher in smokers, compared with ex- ($p < .003$) and never- ($p < .0001$) smokers and in ex- vs. never-smokers ($p = .014$) at, respectively, 13.5 ± 0.7, 11.3 ± 1.0, and 8.7 ± 0.3 μg/g creatinine. Isop$F_2\alpha$ was higher in smokers, compared with ex- ($p = .007$) and never-smokers ($p < .0001$) and in ex- vs. never- smokers ($p = .002$) at, respectively, 1.1 ± 0.10; 0.74 ± 0.07, and 0.51 ± 0.04 μg/g creatinine. There were significant correlations among all three nucleic acid adducts and between Isop$F_2\alpha$ and both 5-OHmU and 8-OHdG. Many smokers and ex-smokers had high levels of either 5-OHmU excretion or Isop$F_2\alpha$ excretion, but not both. We conclude that 5-OHmU and Isop$F_2\alpha$ are more discriminating of oxidative stress from tobacco smoke than the other two compounds measured. Whether characteristic patterns of excretion of these indicators forecast differential disease risk should be explored in future research. © 2003 Elsevier Inc.

Keywords—Isoprostanes, Oxidative stress, Smoking, Nucleic acids, Free radicals

INTRODUCTION

The progressive accumulation of unrepaired free radical damage over time is believed to be a major contributor to the aging process [1,2] and to a variety of age-related chronic diseases [3]. Generation of most free radicals is a "side effect" of normal metabolic processes, especially mitochondrial production of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and hydroxyl radical, coincident to oxidative metabolism. ROS damage proteins, lipid membranes, and nucleic acids (DNA and RNA) [4,5], all critical functional components of living cells. Rates of oxidative damage to nucleic acids in vivo can be assessed by measurement of products of oxidative damage to purine and pyrimidine bases excreted in urine [6,7]. Similarly, rates of lipid peroxidation appear to be related to excretion of F_2-isoprostanes, which are endproducts of the nonenzymatic oxidation of arachidonic acid residues [8,9]. Levels of both DNA damage products [10] and 8-epi prostaglandin $F_2\alpha$ (Isop$F_2\alpha$), one of the best-studied F_2-isoprostanes [11], vary considerably among healthy human subjects [7]. Based on prior studies, it appears that a variety of factors, including smoking, oxygen consumption, and inflammatory disease, modulate oxidative stress status, whereas diet, energy restriction and antioxidant supplements seem to have relatively little effect [6,12].

In one study [7], no significant correlation of Isop$F_2\alpha$ levels with levels of DNA damage products was observed, leading the authors to conclude that no single parameter will be a satisfactory index of overall state of oxidative stress. Thus, it is likely that, in order to assess

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effects of candidate therapies designed to reduce oxidative stress, it will be necessary to employ assays that reflect ongoing rates of oxidative damage to all three critical tissue components. In the study reported below, we compared levels of three nucleic acid-damage adducts and Isop$F_2\alpha$, all measured by liquid chromatography/tandem mass spectrometry (LC-MS/MS) in urine samples from smokers, nonsmokers, and ex-smokers, as indicators of oxidative stress status.

MATERIALS AND METHODS

Research subjects

Urine samples, mainly, but not exclusively, first-morning voided urines, were obtained for measurement of DNA and lipid damage adducts from 234 generally healthy, community-dwelling men ($n = 113$) and women ($n = 121$) of whom 80 were currently smoking at least 10 cigarettes per day (smokers), 96 had no history of smoking tobacco (never-smokers), and 58 had previously smoked, but denied tobacco use in the previous 3 years (ex-smokers). Each group was approximately evenly divided between men and women. The mean age (±SEM) of all subjects was 47.6 ± 0.8 (range, 19–80) years and of smokers, ex-smokers, and nonsmokers, respectively 45.9 ± 1.4, 50.0 ± 1.7, and 47.4 ± 1.3 ($p = .18$) years. Measurements of Isop$F_2\alpha$ were made on a subset ($n = 214$) of the above samples, 72 smokers, 54 ex-smokers, and 88 never-smokers. Subjects were recruited from the greater Phoenix community via advertisements. The research protocol was approved by the Arizona State University Institutional Review Board and subjects gave written informed consent to participate.

Analytical methodologies

Urine samples were aliquoted and stored at −20°C until analysis. Urine creatinine was measured using standard clinical methodology on a Synchron Clinical System LX20 (Beckman Coulter, Fullerton, CA, USA). Thawed samples were mixed for a few seconds on a vortex shaker and centrifuged at 8000 × *g* for 3 min to precipitate solids, and supernatants were separated and analyzed as follows.

Determinations of nucleic acid damage adducts and Isop$F_2\alpha$ were carried out using LC-MS/MS on a system consisting of three Shimadzu LC-10AD pumps, a Shimadzu degasser (Shimadzu Scientific Instruments, Columbia, MD, USA), and a Perkin Elmer autosampler (Perkin Elmer LLC, Norwalk, CT, USA) directly interfaced with a triple-stage quadrupole mass spectrometer (API2000, Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray ionization source. Nitrogen was used as the collision gas. Different ionization methods were used to measure oxidized nucleosides (positive electrospray) and 8-iso-Isop$F_2\alpha$ (negative electrospray). The targeted compounds were measured under multiple reaction-monitoring (MRM) mode. Instrument control, data acquisition, and data analysis were carried out with Analyst software (Applied Biosystem). A seven point linear calibration curve was established for each analysand using both internal and external standards over a range of 0–60 ng/ml for nucleosides and 0–40 ng/ml for Isop$F_2\alpha$. A seven level calibration standard was run at the beginning and the end of each set of unknown samples. Two quality-control samples, made by spiking standards into urine, were injected at the beginning, the end, and after every 10 samples to monitor inter- and intra-day assay accuracy and precision.

High-pressure liquid chromatography (HPLC) grade organic solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). 8-hydroxy-2′-deoxyguanosine (8-OHdG), 5-hydroxymethyl-2′-deoxyuridine (5-OHmU), and 8-hydroxyguanosine (8-OxoG) standards were obtained from Berry & Associates, Inc. (Ann Arbor, MI, USA). The isotope O^{18} labeled 8-OHdG (O^{18}-8-OHdG) was a gift from the National Institutes of Health (Bethesda, MD, USA). The Isop$F_2\alpha$, and isotope labeled 8-iso-prostaglandin-d4 (Isop$F_2\alpha$-d4) standards were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Stock solutions of all standards were prepared by dissolution in either water (for nucleosides) or ethanol (for Isop$F_2\alpha$), aliquoted into small vials, and stored at −70°C.

To determine oxidized nucleosides, all samples were spiked with 4 ng of O^{18}-8-OHdG or 20 ng of Trifluoro-deoxyribonucleoside in each 100 ul of urine supernatant or calibration standard as internal standard. After gentle mixing, 10 μl of standard or urine sample were injected onto a YMC ODS-AQ column (2.0 × 50 mm, 3 μm particle size; Waters, Milford, MA, USA) with an identical guard column (2.0 × 10 mm, 3 μm). Samples were delivered at a flow rate of 200 μl/min with an injection volume of 10 μl. The mobile phases consisted of 10 mM ammonium acetate adjusted to pH 4.2 with formic acid (A) and methanol (B). The HPLC separation was carried out with a solvent gradient program of: 95% A at time 0, a linear decrease to 50% A at 6.0 min, hold for 30 s, drop to 0% A within 30 s, then increase from 0 to 95% A within 1 min. A switch valve was used to regulate, so that only the components eluted out between 2 and 7.5 min were injected into the mass spectrometer. Thirty second equilibration times were used for each sample and the total HPLC running time was 8.5 min.

Under MRM detection mode, ion pairs of 284/168, 259/125, 300/168, and 286/170 were used to detect 8-OHdG, 5-OHmU, 8-OxoG, and O^{18}-8-OHdG, respectively. Trifluoro-deoxyribonucleoside was also evaluated

Table 1. Quality Control Determinations for Known Additions of 8-OHdG, 5-OHmU, and 8-OxoG Standards

	8-OHdG	5-OHmU	8-OxoG
Urine unspiked			
Measured (ng/ml)	3.5 ± 0.3	3.4 ± 0.2	4.4 ± 0.2
Urine spiked with 4 ng/ml			
Measured (ng/ml)	7.4 ± 0.3	7.2 ± 0.2	8.4 ± 0.5
Recovery (%)	97.8	95.6	99.8
Urine spiked with 10 ng/ml			
Measured (ng/ml)	13.0 ± 0.3	13.0 ± 0.9	14.1 ± 0.9
Recovery (%)	95.8	97	97.6
Urine spiked with 20 ng/ml			
Measured (ng/ml)	22.8 ± 0.9	22.8 ± 0.9	23.7 ± 0.9
Recovery (%)	96.8	97.3	96.4

8-OHdG = 8-hydroxy-2′-deoxyguanosine; 5-OHmU = 5-hydroxymethyl-2′-deoxyuridine; 8-OxoG = 8-hydroxyguanosine.

as a potential internal standard, due to the difficulty of obtaining the isotope-labeled nucleosides. Unless otherwise indicated, all of the test results were based on O^{18}-8-OHdG as internal standard. The mass spectrometer was optimized in the MRM mode by diffusing 2 mg/l of each individual nucleoside standard solution.

Method detection limits, determined at the 3× the signal-to-noise ratio obtained using standard solutions containing 0.1–15 ng/ml of each targeted nucleosides, were 0.8, 0.5, and 0.7 ng/ml for 8-OHdG, 5-OHmU, and 8-OxoG, respectively. The assays were linear over the range of 10 pg to 1 ng for each nucleoside. The accuracy and reproducibility of the procedure were estimated by spiking urine samples with three levels of nucleoside standards: 2, 10, and 20 ng/ml. Table 1 shows means and standard deviations of independent measurements of each of the three nucleosides performed on unspiked and each spiked urine sample on 3 different days. Recoveries were consistently in excess of 96% and coefficients of variance (CV) ranged from 2% to 9% with average CVs for unspiked, low-, mid-, and high-dose spiked urines of 6%, 4%, 5%, and 4% respectively.

We determined Isop$F_2\alpha$ according to our previously published method [13]. Briefly, 1ml of urine supernatant was transferred into a glass tube, spiked with 10 ng of internal standard (8-iso-Isop$F_2\alpha$-d4), and diluted with 1 ml of deionized (DI) water. Samples were purified through a C18 solid phase extraction (SPE) cartridge on an automated SPE workstation (RapidTrace; Zymark, Hopkinton, MA, USA). Varian's Bond Elut C18 cartridges (3 cc/500 mg; Varian, Harbor City, CA, USA) were solvated with 5 ml of ethanol and equilibrated with 5 ml of DI water. Samples were loaded and washed sequentially with 5 ml of water, 5 ml of ethanol:water (5:95 v/v), and 1 ml of hexane and eluted with 2 ml of ethyl acetate. The eluent was evaporated to dryness under a steam of nitrogen gas and reconstituted in 50 μl of acetonitrile:water (20:80 v/v) solution.

Ten microliters of reconstituted sample was injected onto the same HPLC column as above. Mobile phases were methanol:acetonitrile (5:95 v/v) (A) and 2 mM ammonium acetate (B). HPLC separation was carried out with a solvent gradient program of: 15% A at time 0, a linear increase to 70% A at 6 min, a linear increase to 100% A at 8 min, then a linear decrease from 100% to 15% A within 1 min. The ion pairs of m/z 353/193 and m/z 357/197, were used to monitor Isop$F_2\alpha$, and Isop$F_2\alpha$ -d4, respectively.

Statistical analyses

Raw data were compiled in a Statview 5.0 for Macintosh (SAS Institute, Cary, NC, USA) database. Means, standard deviations, and ranges were calculated and analyses of covariance and regression analyses were performed using Statview 5.0 routines. Because distribution analyses revealed that values for all four measures were log-normally distributed, analyses of variance to explore effects of smoking status and sex were conducted on log-transformed data. Mean levels of each of the analytes in each of three groups were compared by analysis of variance (ANOVA) with post-hoc three-way comparisons by the Bonferroni-Dunn test, correcting for multiple comparisons. In addition we employed linear regression analyses with correlation coefficients (Pearson's r) and p values for significance of slope to assess effects of age and the covariance of each of the separate analytes with one another in all subjects pooled and within groups, defined as above. Finally, distributions of subjects by group into ≥90th percentile and <90th percentile categories for 5-OHmU and Isop$F_2\alpha$ excretion were tested for significance by the χ^2 procedure.

RESULTS

Effects of age and sex

Comparisons of values (mean ± SEM) for each of the oxidative stress indicators in men vs. women revealed that only Isop$F_2\alpha$ excretion was significantly different, being higher in women than in men (0.92 ± 0.07 vs. 0.62 ± 0.05 μg/g creatinine; p = .0004). When smoking groups were compared separately, the sex difference in Isop$F_2\alpha$ persisted in the smokers (p = .01) and the never-smokers (p = .0005) but did not reach statistical significance in the ex-smokers (p = .11). The excretion of 5-OHmU was also significantly greater in women vs. men in the never-smoker group (0.96 ± 0.02 vs. 0.85 ± 0.02 μg/g creatinine; p = .0006). There were no significant sex differences for 8-OHdG or 8-OxoG.

Table 2. Comparisons of Urinary Excretion of Three DNA Oxidative Damage Adducts in Smokers, Ex-smokers, and Nonsmokers Measured by LC-MS/MS

Groups	Assay (n)	8-OHdG (μg/g Cr)	5-OHmU (μg/g Cr)	8-OxoG (μg/g Cr)
Smokers	80	6.9 ± 0.3	13.5 ± 0.7*†	9.9 ± 0.6
Ex-Smokers	58	6.6 ± 0.6	11.3 ± 1.0‡	9.3 ± 0.8
Never-smokers	96	6.3 ± 0.2	8.7 ± 0.3	8.4 ± 0.5

LC-MS/MS = liquid chromatography/tandem mass spectrometry.
* $p < .0001$ vs. Never-smokers.
† $p < .003$ vs. Ex-smokers.
‡ $p = .014$ vs. Never-smokers.

Linear regression of each of the measures against age revealed that both 8-OHdG ($r = 0.141$, $p = .03$) and 8-OxoG ($r = 0.153$, $p = .02$) excretion tended to increase with age in all subjects considered together. Urinary excretion of 5-OHmU and IsopF$_2\alpha$ did not appear to be affected by age. When smoking groups were compared separately, there were significant increases with age in 8-OHdG only in smokers ($r = 0.337$, $p = .01$), and in 8-OxoG only in ex-smokers ($r = 0.294$, $p = .025$), but nonsignificant trends in smokers for 8-OxoG ($p = .13$) and ex-smokers for 8-OHdG ($p = .07$).

Effects of smoking history

Mean values in each smoking group for each of the three DNA damage adducts are shown in Table 2. Ex-smokers had levels of 8-OHdG intermediate between, but not significantly different from, those of current smokers ($p = .75$) and never-smokers ($p = .63$). Compared with smokers, never-smokers had lower levels of 8-OHdG, but not significantly so ($p = .13$). All comparisons were nonsignificant when sexes were analyzed separately (data not shown). Levels of 5-OHmU were highest in current smokers, intermediate in ex-smokers, and lowest in never-smokers. Smokers differed significantly from never-smokers ($p < .0001$) and from ex-smokers ($p = .003$), and ex-smokers differed significantly from never-smokers ($p = .014$). All group differences for 5-OHmU remained significant among smoking groups in men considered separately, but in women only never-smokers and current smokers differed significantly (0.96 ± 0.02 vs. 1.12 ± 0.04 μg/g creatinine, $p = .0007$). Urinary 8-OxoG was somewhat higher in smokers than in never-smokers, with ex-smokers again having intermediate values, but values for this adduct did not differ significantly among the three groups pooled or in men and women considered separately (data not shown).

Urinary IsopF$_2\alpha$ levels in the smokers, ex-smokers, and never-smokers were, respectively, 1.10 ± 0.10, 0.74 ± 0.07, and 0.51 ± 0.04 μg/g creatinine. By three-way ANOVA for IsopF$_2\alpha$, smokers differed from never-smokers ($p < .0001$) and ex-smokers ($p = .006$), which latter groups also differed from one another ($p = .002$). Comparing smoking groups by sex, differences between male ex-smokers and current smokers were no longer statistically significant (0.58 ± 0.07 vs. 0.91 ± 0.10μg/g creatinine, $p = .05$, not significant after Bonferroni-Dunn correction), whereas female ex-smokers' somewhat higher levels of IsopF$_2\alpha$ excretion, did not differ significantly from never-smokers (0.88 ± 0.12 vs. 0.65 ± 0.06μg/g creatinine, $p = .10$).

Interrelationships of different measures

Results of linear regression analyses relating each of the indicators of oxidative damage with each of the others in all subjects pooled are shown as a correlation matrix in Table 3. There were significant positive relationships of 8-OHdG with 5-OHmU (Fig. 1A), 8-OxoG, and IsopF$_2\alpha$ (Fig. 2A). Levels of 5-OHmU also correlated both with 8-OxoG (Fig. 1B) and IsopF$_2\alpha$ (Fig. 2B) However, 8-OxoG did not co-vary significantly with IsopF$_2\alpha$.

Table 3. Correlation Matrix: r and p Values for Urinary Nucleic Acid Damage Adducts and Isoprostane F$_2\alpha$

Measurement	8-OHdG	5-OHmU	8-OxoG
5-OHmU	0.508 ($p < .0001$)		
8-OxoG	0.374 ($p < .0001$)	0.493 ($p < .0001$)	
IsopF$_2\alpha$	0.232 ($p = .0006$)	0.355 ($p < .0001$)	0.097 ($p = .16$)

8-OHdG = 8-hydroxy-2′-deoxyguanosine; 5-OHmU = 5-hydroxymethyl-2′-deoxyuridine; 8-OxoG = 8-hydroxyguanosine.

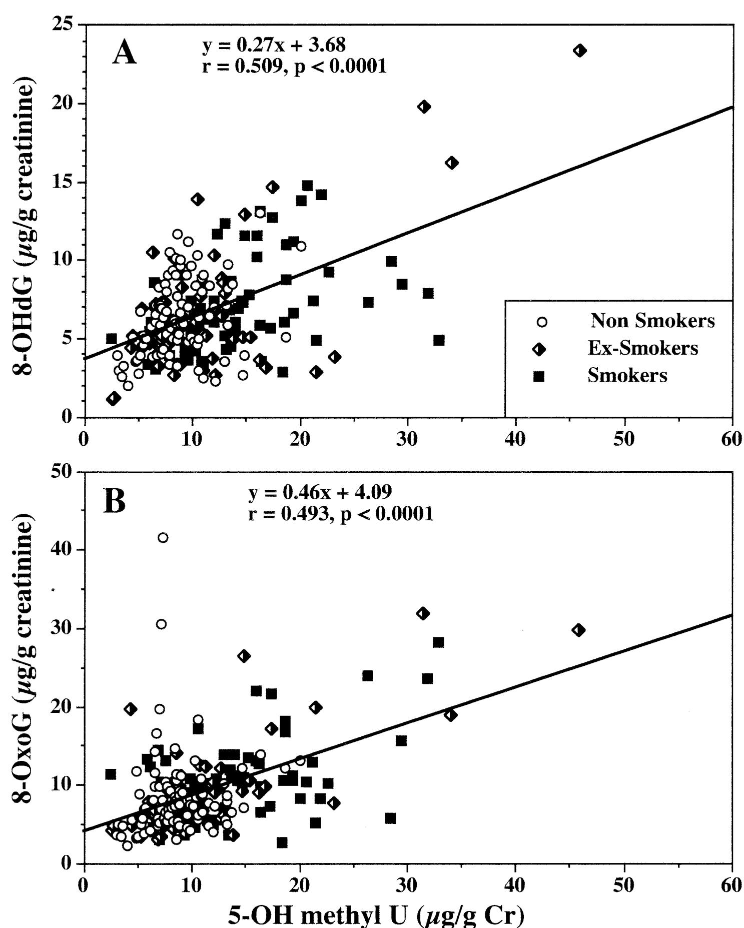

Fig. 1. Bivariate plots and linear regression analyses of levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) (panel A) and 8-hydroxyguanosine 8OxoG (8-OxoG) (panel B) vs. 5-hydroxymethyl-2′-deoxyuridine (5-OHmU) in random urine samples from smokers, never-smokers, and ex-smokers. Levels of 8-OHdG and 8OxoG correlate significantly with those of 5-OHmU.

Because 5-OHmU and Isop$F_2\alpha$ discriminated best among smoking groups and 5-OHmU was more strongly correlated with Isop$F_2\alpha$ than were the other two nucleic acid adducts, we examined their relationships more closely. As can be seen in Fig. 2B, subjects with the very highest levels of Isop$F_2\alpha$ (90th percentile = >1.55 μg/g creatinine) and those with the highest levels of 5-OHmU (90th percentile = >18.4 μg/g creatinine) were predominantly smokers. Of the 37 subjects in the 90th percentile for excretion of either 5-OHmU or Isop$F_2\alpha$, 26 were smokers, 8 were ex-smokers, and 3 were never-smokers ($p < .0001$). However, only 6 (16%) were excreting high levels of both compounds, which is about half the number (12.5) of subjects expected if high excretion of both compounds were distributed at random ($p < .001$). Thus, of subjects with the highest 5-OHmU excretion, most (73%) did not have high Isop$F_2\alpha$ and of subjects with the highest Isop$F_2\alpha$ excretion, most (71%) did not have high 5-OHmU.

DISCUSSION

A continually growing body of data implicates damage to cells, tissues, and extracellular components by ROS and RNS in the pathogenesis of a variety of chronic age-related diseases including cardiovascular disease (CVD) [14]; cancers [15,16]; neurological diseases, such as Parkinson's [17] and Alzheimer's [18,19] diseases; diabetes [20]; and the aging process itself [1,2]. Epidemiologic evidence suggests that diets high in antioxidants may decrease incidence of cancer [21] and CVD [22], but trials of antioxidant supplements have largely been disappointing in this regard [23,24]. It is the authors' contention that candidate antioxidant interventions aimed at reducing rates of CVD or cancer should undergo preliminary evaluation to determine if they actually reduce rates of oxidative damage in humans. As discussed by Halliwell [21], such evaluations should employ multiple measures of oxidative damage to different tissue components (nucleic acids, lipid, and protein), in order to give a more complete profile of antioxidant action.

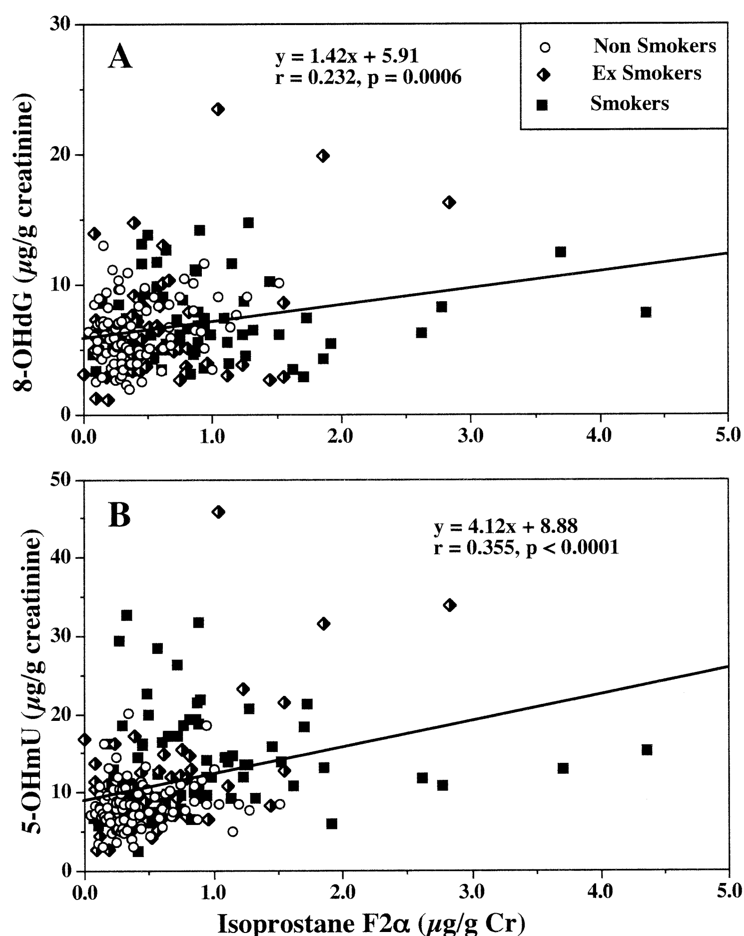

Fig. 2. Bivariate plots and linear regression analyses of levels of the DNA damage adducts, 8-hydroxy-2′-deoxyguanosine (8-OHdG) (panel A) and 5-hydroxymethyl-2′-deoxyuridine (5-OHmU) (panel B) vs. 8-iso-prostaglandin $F_2\alpha$ 8-epi prostaglandin $F_2\alpha$ (Iso$pF_2\alpha$) in random urine samples from smokers, never-smokers, and ex-smokers. Levels of both 5-OHmU and 8-OHdG correlate significantly with those of Iso$pF_2\alpha$.

It is also possible that pathogenesis of different diseases will be characterized by different patterns of oxidative damage. To assess this possibility, we applied new methods, utilizing high-throughput LC-MS/MS to examine levels of three markers of nucleic acid oxidative damage, 8-OHdG, 5-OHmU, and 8-OxoG, and the isoprostane, Iso$pF_2\alpha$ [13] in stored urine samples obtained from patients currently smoking, patients who had never smoked, and patients with variable smoking histories, but no tobacco use for at least the previous 3 years.

A variety of studies [25,26] have shown cigarette smoking to be associated with increased oxidative damage to DNA and lipid in cells and tissues. Mechanisms of oxidative damage due to smoking may include an increase in metabolic rate and hence enhanced mitochondrial ROS production [25] and also the direct actions of a variety of active oxidants and pro-oxidants present in tobacco smoke [27]. In addition, smoking activates inflammatory/immune cells [28] such as monocytes, which have been shown to produce ROS that may contribute to local oxidation of lipids, lipoproteins, and cell membranes [29]. Thus, smokers provide a useful model for evaluating and validating methods for quantification of oxidative stress. Moreover, it is likely that oxidative damage from smoking is an important mediator of smoking-related diseases. For example, cigarette smoking is a major risk factor for CVD of the same order as elevated low-density lipoprotein (LDL) cholesterol [30] and C-reactive protein [31]. One probable mechanism by which smoking increases cardiovascular risk is by increasing oxidation of LDL cholesterol [32], which promotes scavenging uptake of oxidized LDL particles by macrophages and accelerates atherogenesis [33]. Cigarette smoking is also known to be a strong risk factor for lung and other cancers [34]. Because mutations resulting from oxidative damage to DNA are believed to play an important role in carcinogenesis

[25], oxidative stress produced by smoking is almost certainly a critical link between tobacco use and cancer risk.

Determination of urinary excretion of 8-OHdG has long been the "gold standard" assay for estimating rates of random oxidative damage to DNA in intact organisms because this adduct is formed in relatively large quantities in vivo [35], is excised from DNA by excision repair, and excreted unchanged in the urine [36]. Various nucleic acid damage adducts, including 8-OHdG [6,7] and 5-OHmU [37], have been shown to be elevated in conditions related to high oxidative stress. The production of 5-OHmU may have particular relevance to carcinogenesis, as studies have found elevated titers of autoantibodies to this DNA oxidation product in patients with lung [38] and other [39] cancers. Most prior studies measuring urinary or plasma nucleic acid damage adducts as indicators of oxidative stress status have not included 5-OHmU because the required derivatization step in the standard gas chromatography/MS method degrades this compound. Our LC-MS/MS method does not require derivatization, so that we were able to quantify 5-OHmU without difficulty.

The F_2-isoprostanes are formed in lipid membranes and particles by spontaneous oxidation of arachidonic acid and the urinary excretion rates of isoprostanes have been shown to be related to levels of oxidative stress and lipid peroxidation in various states of health and disease [40,41], including cigarette smoking [42]. In the current study, smokers had higher urinary levels of 8-OHdG, 8-OxoG, 5-OHmU, and Isop$F_2\alpha$ than never-smokers, but only 5-OHmU and Isop$F_2\alpha$, differed significantly. The 8-OHdG, 8-OxoG, 5-OHmU and Isop$F_2\alpha$ values were intermediate in ex-smokers, but again, only the latter two compounds differed significantly (from both smokers and never-smokers). In prior studies examining urinary excretion of nucleic acid oxidative damage markers [42], smokers excreted 35% to 50% more 8-OHdG than did nonsmokers, results inconsistent with the 9% difference we observed. In a longitudinal study of smoking cessation [43], urinary 8-OHdG excretion decreased by approximately 16% during the first 4 weeks of abstinence, and then remained stable up to 26 weeks later. In the latter study, 8-OHdG excretion levels remained higher in ex-smokers than those previously reported for never-smokers, a result consistent with findings in the current study. We estimated values for microgram 8-OHdG excreted per gram creatinine in prior studies, in which total or per kilogram body weight 24-h excretion was reported, by assuming a body weight of 70 kg and 1.5 g/d of creatinine excretion per 24 h. Estimates range from 4.5 [42] to 6.2 [43] μg/g creatinine in smokers, values in the same range as the 6.9 μg/g creatinine we observed in the current study. However, as noted above, corresponding levels (3.0 and 4.0 μg/g creatinine, respectively) were lower in the non- or ex-smokers in the prior studies.

Our observation that Isop$F_2\alpha$ excretion per gram of creatinine was 33% greater in female than in male smokers and ex-smokers probably reflects greater relative lipid oxidation and a lower creatinine denominator in women due to their higher percent body fat and lesser lean body (muscle) mass. It has been previously observed that isoprostane excretion is greater in women [44] and positively associated with body mass index in both sexes [45]. We measured greater concentrations of urinary creatinine in men than women (138 ± 6 vs. 111 ± 6 mg/ml) and found that absolute concentrations of Isop$F_2\alpha$ per milliliter of urine, although still lower in men than in women (79 ± 6.8 vs. 102 ± 12.0 μg/ml) were no longer significantly so (p = .09). Alternatively this finding may indicate greater susceptibility of lipid to oxidation during smoking in women. It is also possible, but less likely, that the observed difference was produced by differential smoking behavior between the sexes. Further research comparing measured body composition to Isop$F_2\alpha$ excretion and quantifying cigarette consumption may help clarify this relationship.

We found urinary Isop$F_2\alpha$ levels to be about 50% higher in smokers than in nonsmokers, with intermediate levels observed in former smokers. In one previous study [46], Isop$F_2\alpha$ levels were more than 2-fold higher in urine of smokers than in age- and sex-matched nonsmokers. Smoking cessation led to a rapid decrease in both urinary and plasma Isop$F_2\alpha$, with urinary Isop$F_2\alpha$ decreasing to a plateau, after 14–21 days at levels still somewhat, but not significantly, higher than those of nonsmokers. In a similar study [47] urinary Isop$F_2\alpha$ excretion was also about 2-fold higher in smokers than nonsmokers with a dose–response relationship in the smokers between urinary Isop$F_2\alpha$ levels and number of cigarettes smoked. Smoking cessation for 3 weeks produced a decrease but Isop$F_2\alpha$ level remained considerably greater than that measured in nonsmokers.

In contrast with a prior study [7], we found significant correlations, not only of rate of excretion of nucleic acid adducts with one another, but also of 8-OHdG and 5-OHmU with Isop$F_2\alpha$. Of the DNA damage adducts measured, 5-OHmU correlated most strongly with levels of Isop$F_2\alpha$, which relationship was strongest in smokers, present in ex-smokers, but not observed in never-smokers. These correlations suggest that the compounds measured do indicate overall oxidative stress status.

The observation that excretion of oxidative damage markers was higher in ex-smokers than in never-smokers could be explained by a continuing higher level of ongoing oxidative stress in former smokers due to long-term chronic damage to cells and tissues in the lungs and elsewhere, possibly to ongoing inflammatory processes (e.g., chronic bronchitis). It has been reported that inflammatory markers remained elevated up to 1 year after smoking cessation [48], possibly due to persistence of

residual infection in the bronchial tree [49], and inflammation is itself a source of increased ROS production [50]. A second possible explanation is persistence of certain damage products in tissue for long periods of time, due to less-efficient recognition and removal by repair systems. The former explanation is more likely to explain the higher Isop$F_2\alpha$ levels we observed in ex-smokers, whereas the latter mechanism could be responsible for the higher levels of 8-OHdG excretion in this group. Consistent with the latter hypothesis is our observation that 8-OHdG and 8-OxoG excretion tended to increase with age in smokers and ex-smokers, suggesting that these damage products may persist and accumulate, but remain subject to recognition and removal by repair mechanisms. Also consistent with this concept is the finding by Asami et al. [26] that higher levels of 8-OHdG are found in DNA extracted from the lungs of ex-smokers compared with nonsmokers.

Limitations of the current study include its lack of longitudinal data, and incomplete information on smoking history of the patients whose samples we examined. Nonetheless, our results suggest that urinary excretion of 5-OHmU and Isop$F_2\alpha$ reflect current oxidative stress status better than that of 8-OHdG or 8-OxoG. Thus, the former two markers may prove more useful as indicators of acute response to candidate antioxidant interventions. The finding that 8-OHdG and 8-OxoG excretion increased progressively with age in smokers suggests that these markers may be better indicators of long-term cumulative DNA damage and could be utilized to assess persisting residual damage or damage history. Future research should assess relationships of these markers in states of oxidative stress other than cigarette smoking.

In summary, novel aspects of the present study include: the use of high-throughput LC tandem MS to measure urinary excretion of three different nucleic acid damage adducts and an isoprostane in the same human subjects, providing simultaneous assessment of two different classes of oxidative stress; the finding that 5-OHmU discriminates better between smokers and nonsmokers than 8-OHdG or 8-OxoG; and the observation that smokers and ex-smokers can be divided into those with high 5-OHmU excretion or high Isop$F_2\alpha$ excretion, with relatively few excreting the highest levels of both indicators. This latter result suggests that smoking produces different patterns of oxidative stress in different individuals. Whether these differences are related to genetic factors mediating endogenous antioxidant protective mechanisms, differences in diet and antioxidant intake, or both, and, perhaps more important, whether these patterns may indicate differential disease risks, with high 5-OHmU predicting cancer and high Isop$F_2\alpha$ forecasting cardiovascular disease, should be the subjects of future research.

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ABBREVIATIONS

CVD—cardiovascular disease
Isop$F_2\alpha$—8-iso-prostaglandin $F_2\alpha$ 8-epi prostaglandin $F_2\alpha$
LC-MS/MS—liquid chromatography/tandem mass spectrometry
8-OHdG—8-hydroxy-2′-deoxyguanosine
5-OHmU—5-hydroxymethyl-2′-deoxyuridine
8-OxoG—8-hydroxyguanosine
RNS—reactive nitrogen species
ROS—reactive oxygen species