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Ascorbylated 4-hydroxy-2-nonenal as a potential biomarker of oxidative stress response

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Abstract

Oxidative stress, resulting from the generation of reactive oxygen species, contributes to the development of a multitude of age-related diseases. Current methods of assessing oxidative stress levels range from the detection of lipid peroxidation products, such as F_2-isoprostanes and malondialdehyde, to monitoring the redox status of glutathione. While useful, traditional biomarkers of oxidative stress are not without their drawbacks, including low in vitro concentrations and possible artifact formation. In the present study, we utilize liquid chromatography coupled with tandem mass spectrometry for investigation into the use of a novel compound, ascorbylated 4-hydroxy-2-nonenal, as a potential biomarker of oxidative stress.

Keywords: Oxidative stress; Biomarker; 4-Hydroxy-2-nonenal; Vitamin C; Ascorbic acid; Lipid peroxidation; Mass spectrometry

1. Introduction

Oxidative stress results from an imbalance between production of free radicals and antioxidant defenses [1]. With the cells' natural antioxidant mechanisms overwhelmed, the excess reactive oxygen species may cause radical-mediated damage to biomolecules [2]. Polyunsaturated fatty acids (PUFAs) are especially susceptible to oxidation and readily form lipid hydroperoxides that are largely reduced to their hydroxyl analogs by glutathione peroxidase in the presence of glutathione [1]. A small fraction of the lipid hydroperoxide pool may decompose non-specifically and ultimately give rise to cytotoxic and genotoxic α,β-unsaturated aldehydes and other lipid peroxidation (LPO) products [3]. These aldehyde species are soft electrophiles, which exhibit toxicity by covalently modifying nucleophilic moieties of proteins (especially histidine, cysteine, and lysine residues) [4–7] and DNA (primary amines) [8,9]. 4-Hydroxy-2-nonenal (HNE), 4-oxo-2-nonenal (ONE) and malondialdehyde (MDA) are some of the better characterized LPO products [3].

Reactive aldehyde species, generated from oxidative stress-induced peroxidation of lipids, are both cytotoxic and genotoxic [10,11]. Additionally, these compounds have been associated with the development and progression of inflammatory diseases such as atherosclerosis, lupus and Alzheimer's disease [12–17]. Consequently, the development of biomarkers of oxidative stress is of great interest. Current biomarkers include concentration determinations of LPO products such as F_2-isoprostanes, free MDA and HNE, and their adducts of lysine, glutathione and 2′-deoxyguanosine [18–34]. While much success has been achieved using this approach, chemical stability is a concern. A second approach of assessing oxidative stress is to monitor the redox status of glutathione, though artifacts may pose problems due to autooxidation of glutathione [35–39].

The role of vitamin C in oxidative stress is incompletely understood. While ascorbic acid is best known as an antioxi-

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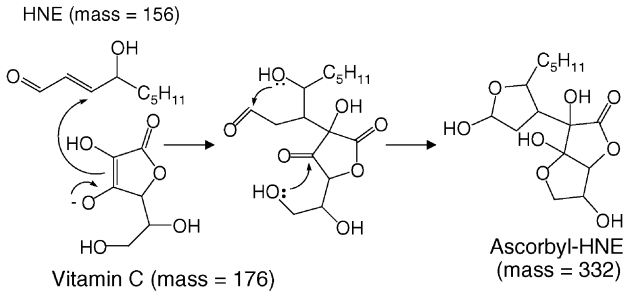

Fig. 1. Formation of the ascorbyl–HNE conjugate. Ascorbic acid functions as a nucleophile and forms a conjugate with 4-hydroxy-2-nonenal via Michael chemistry.

dant, we have recently shown that vitamin C can function as a biological nucleophile, demonstrated by the formation of a Michael adduct with the LPO product, HNE (Fig. 1) [40]. Using liquid chromatography tandem mass spectrometry, we confirmed the presence of the ascorbyl–HNE conjugate and other ascorbylated LPO products in human plasma [40]. Based on our previous results [40], we hypothesized that the ascorbyl–HNE conjugate could be used as a biomarker of oxidative stress. Because it is well known that smoking induces oxidative stress [27,41–44], we used cigarette smoking as an in vivo model to determine the value of the ascorbyl–HNE conjugate as a biomarker of oxidative stress in comparison with F_2-isoprostanes.

2. Experimental

2.1. Chemicals

The ascorbyl–HNE adduct and the internal standard, ascorbylated 2-octenal, were synthesized as described previously [40]. HPLC-grade acetonitrile was from Burdick and Jackson (Morristown, NJ). All other chemicals were obtained through Sigma Chemical (St. Louis, MO).

2.2. Chromatography

All HPLC experiments were performed using a C18 column (250 mm × 1 mm, 4 μm, Synergi Max RP; Phenomenex, Torrance, CA) with a flow rate of 50 μl/min. The column was interfaced directly to the mass spectrometer. Solvent A was 10 mM ammonium acetate and 0.1% (v/v) formic acid in MilliQ water (pH 4.0). Ammonium acetate was added to the solvent to aid in the ionization process, as not all species analyzed were efficiently protonated. Solvent B was acetonitrile. A linear gradient, 25% B to 85% B over 45 min, was used.

2.3. Mass spectrometry

Mass spectrometry experiments were conducted on a Perkin-Elmer Sciex API III Plus triple quadrupole mass spectrometer, operated in positive ion mode and equipped with an electrospray ion source (Concord, Canada). Nitrogen was used as the curtain gas and zero air was used as the sheath gas. For collision-induced dissociation experiments, argon was used as the collision gas, with a collision energy of 15 eV. For product ion and precursor ion scanning, a scan rate of 2 s was used.

Table 1
Summary of subject characteristics[a]

Parameter	Nonsmokers ($n = 10$)	Smokers ($n = 10$)
Age (years)	19.5 ± 2.5	21.0 ± 1.7
Height (m)	1.69 ± 0.15	1.76 ± 0.13
Weight (kg)	63.6 ± 14.4	68.0 ± 9.0
BMI (kg/m^2)	22.2 ± 3.2	22.0 ± 2.2
Dietary supplements	None	None
Cigarettes/day	0	10.6 ± 3.7
Urinary cotinine (ng/ml)	27 ± 13	2587 ± 1615

[a] All data are reported as mean ± S.D.

2.4. Study participants

Plasma samples were obtained from subjects who participated in a recently completed study at the Linus Pauling Institute (Table 1) [42]. The Institutional Review Board at Oregon State University approved the study protocol, and all participants provided written consent prior to enrollment. Participants were recruited on the basis of normal lipid status (total cholesterol <200 mg/dL; triglycerides <200 mg/dL), age (18–35 years), non-nutritional supplement use for greater than six months, and exercise status (<5 h/w of aerobic activity). Smokers were selected if they smoked >10 cigarettes/d, and smoking status of participants was verified by the measurement of urinary cotinine (Diagnostics Products Corp, CA). As suggested by the manufacturer, a urinary cotinine concentration of >500 ng/mL was used as a cutoff to confirm smoking status.

2.5. Sample collection and handling

A blood sample was obtained from the antecubital vein of each participant after an overnight fast (∼12 h) into blood collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin. Smokers were asked to refrain from smoking for 1 h prior to blood collection to obviate transient oxidative stress effects. Plasma was separated by centrifugation (500 × g, 15 min, 4 °C; Beckman TJ-6, Palo Alto, CA), aliquoted into cryovials, snap frozen in liquid nitrogen, and then stored at −80 °C until analysis. Urine was collected for 24 h on a single occasion to evaluate urinary cotinine. Aliquots of urine were stored at −80 °C until analysis.

2.6. Analysis of the ascorbyl–HNE conjugate in human plasma

Two hundred microlitre-aliquots of human plasma were acidified with 250 μl of 0.1 M HCl. One ml of water was then

added. Ethyl acetate was used to extract the ascorbyl–HNE conjugate (3 ml × 3 ml). The combined organic layers were then dried under a stream of nitrogen. The residue was redissolved in 65 μl of ethanol and mixed with 65 μl of LC Solvent A. Prior to injection, the samples were centrifuged for 5 min at 10,000 rpm. Injection volumes were 20 μl. Controls for the formation of ex vivo-artifact formation were performed as described previously [40].

A curve allowing for the determination of ascorbylated HNE in human plasma was constructed utilizing liquid chromatography with tandem mass spectrometry operated in multiple reaction monitoring mode (LC–MS/MS-MRM). Varying amounts of the ascorbyl–HNE adduct were mixed with a fixed amount of the internal standard, ascorbyl-octenal, to give 0.5, 1.0, 5.0, 10 and 50 μM of the analyte and 25 μM of the internal standard. Analyte/internal standard response was plotted against analyte concentration. The transitions *m/z* 350 → *m/z* 177 and *m/z* 320 → *m/z* 223 were used for quantitation of the synthetic standard and internal standard, respectively. Injections were done in triplicate, with 20 μl injection volumes.

Plasma samples from 20 subjects, 10 smokers and 10 nonsmokers, were prepared as described above, with the exception that the plasma samples were spiked with internal standard to give a final concentration of 25 μM after reconstituting the residue in 65 μl of ethanol. Thus, the sample work-up method concentrates the analyte relative to the internal standard by a factor of 200/65 = 3.1. The samples were analyzed by LC–MS/MS-MRM and the concentration of the ascorbyl–HNE adduct determined through comparison with a calibration curve. Injections were done in duplicate, using 20 μl-injection volumes.

Statistical analysis was performed using GraphPad Prism (version 4.0) obtained from GraphPad Software (San Diego, CA). An unpaired Student's *t*-test was used for all comparisons between smokers and nonsmokers. Data were considered statistically significant if $p < 0.05$. All data are reported as mean ± SD unless otherwise noted.

2.7. Standard addition curve

Two hundred microlitre-aliquots of a plasma sample were spiked with varying amounts of ascorbyl–HNE adduct to give concentrations of 1.0, 2.0, 4.0 and 6.0 μM. The plasma samples were also spiked with internal standard (ascorbyl-octenal, 25 μM after reconstitution in 65 μl of ethanol). The samples were analyzed by LC–MS/MS using MRM as described above.

3. Results and discussion

3.1. Detection of the ascorbyl–HNE conjugate in human plasma

The structure of the ascorbyl–HNE adduct, prepared chemically from ascorbic acid and HNE, was determined by NMR spectroscopy and ESI-MS/MS analysis in previous work [40]. The presence of the ascorbyl–HNE conjugate in human plasma was established by LC–MS/MS comparison of the synthetic standard with the endogenous conjugate. The retention time of the endogenous conjugate was identical with that of the synthetic standard, which was confirmed by spiking a plasma sample with the standard. Fig. 2 shows that the standard and the endogenous conjugate yield virtually identical daughter ion spectra upon collision-induced dissociation (CID) of the $[M + NH_4]^+$ ion with *m/z* 350. These daughter ions arise from loss of ammonia and water molecules (*m/z* 333 $[MH]^+$, *m/z* 315 $[MH - H_2O]^+$, and *m/z* 297 $[MH - 2H_2O]^+$) and from cleavage of the carbon-carbon bond between the ascorbyl and HNE moieties (*m/z* 177 [ascorbic acid + H]$^+$ and *m/z* 139 $[HNE + H - H_2O]^+$). The differences in the fragment ion intensities between the standard and the endogenous conjugate are due to the low ion yields and the small number of spectral scans obtained from the endogenous conjugate.

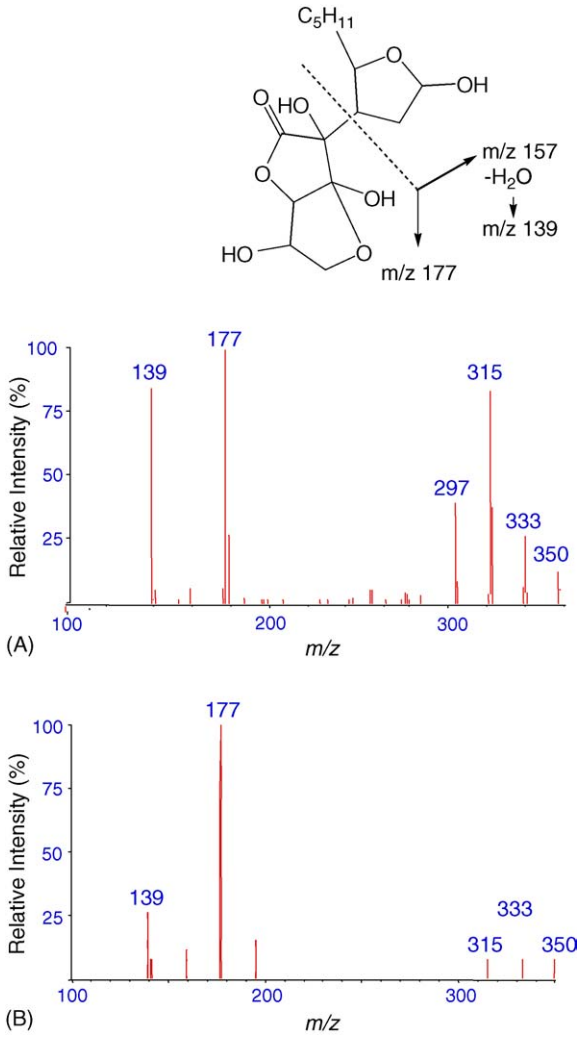

Fig. 2. Tandem mass spectrometry analysis of ascorbylated HNE. MS/MS daughter scans of the *m/z* 350 $[M + NH_4]^+$ ion for (A) the synthetic ascorbyl–HNE adduct and (B) the ascorbyl–HNE conjugate in human plasma.

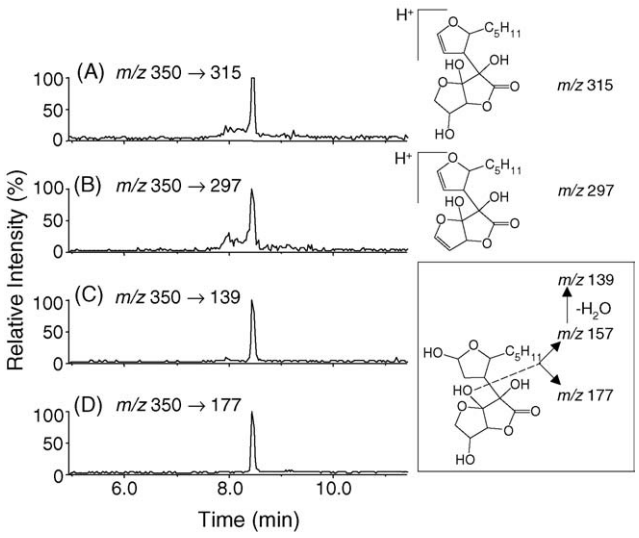

Fig. 3. Liquid chromatography–tandem mass spectrometry analysis with multiple reaction-monitoring of plasma for the presence of ascorbylated 4-hydroxy-2-nonenal. Plasma was extracted and analyzed as described in Experimental. The panels show detection of specific fragment ions, i.e., m/z 315 $[MH - H_2O]^+$ (A), m/z 297 $[MH - 2H_2O]^+$ (B), m/z 139 [hydroxynonenal–H_2O + H]$^+$ (C) and m/z 177 [ascorbic acid + H]$^+$ (D) arising from collisional fragmentation of the quasi-molecular ion with m/z 350 $[M + NH_4]^+$.

Analysis by LC–MS/MS-MRM, based on the MS/MS fragmentation of the ascorbyl–HNE conjugate, allowed for sensitive and selective detection of the ascorbyl–HNE conjugate in human plasma. Fig. 3 shows the ion currents of four diagnostic fragment ions arising from CID of the $[M + NH_4]^+$ ion with m/z 350 in an LC-MRM experiment. The appearance of a single peak matching the retention time of the synthetic adduct indicates that the ascorbyl–HNE conjugate can be detected in human plasma without interference by other plasma constituents, a prerequisite for quantitative analysis of the conjugate in plasma.

3.2. Quantitation of the ascorbyl–HNE conjugate in human plasma

A calibration curve allowing for quantitation of the ascorbyl–HNE conjugate in plasma was constructed. Ascorbyl-octenal was used as an internal standard after it had been confirmed that the compound, or an interfering artifact, was not already present in plasma. Varying amounts of the synthetic ascorbyl–HNE adduct were mixed with a fixed amount of internal standard to give 0.5, 1.0, 5.0, 10 and 50 μM concentrations of the analyte and 25 μM of the ascorbyl-octenal adduct. The ratio of their responses was plotted as a function of the ascorbyl–HNE conjugate concentration (Fig. 4A). Linearity was observed over the entire concentration range. The plasma work-up method results in a 3.1-fold increase of the analyte concentration relative to the internal standard concentration of the calibration solutions, thus justifying the inclusion of the *supra* physiological

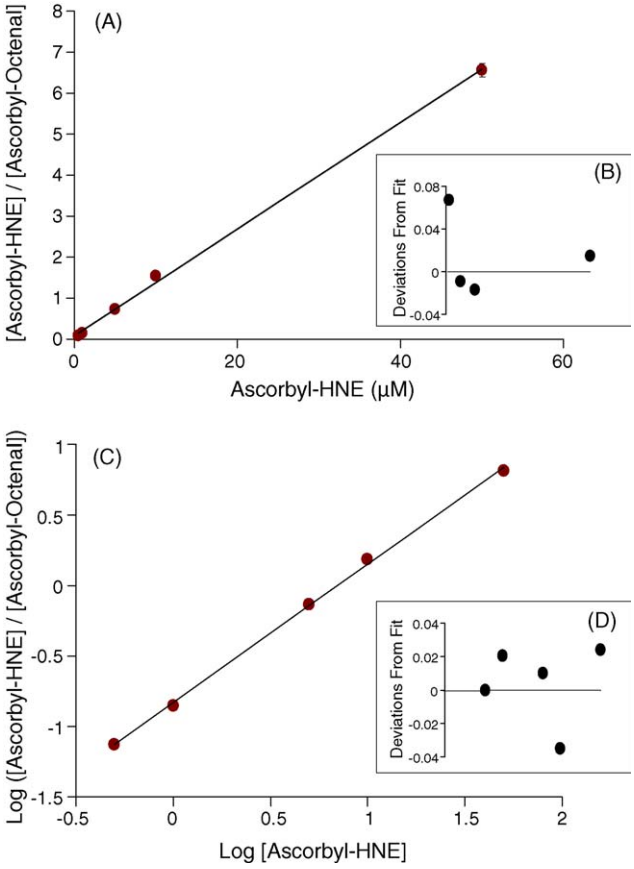

Fig. 4. Calibration curves for the ascorbyl–HNE adduct. Analyte/internal standard response ratios were plotted as a function of analyte concentration. Ascorbylated 2-octenal was used as the internal standard. Each point represents the average of three injections. Error bars indicate mean ± S.D. (A) Linear scale, (C) log scale.

analyte concentrations used in the calibration curve. All analyte/internal standard ratios measured in this study fell within the calibration range of the curve. It was found that the detection limit of the analysis was 0.03 μM ascorbylated HNE in plasma ($S/N > 3$).

A potential problem in using the constructed calibration curve is the systematic increase in variance as the concentration of the ascorbyl–HNE adduct increases. Consequently, the higher concentration points are more influential than the lower concentration points with respect to curve fitting. A plot of the deviations as a function of the fit illustrates this point (Fig. 4B). The magnitude of the deviation is much higher at the lower concentration points, due to the influence of the increased variance of the higher concentration points. The most straight forward way of circumventing this problem is to construct a log–log plot, thereby more evenly distributing the influence each individual point has on the fit of the curve (Fig. 4C). A plot of the deviations as a function of fit for the log–log plot is shown in Fig. 4D. It can be seen that the deviations are not systematic, indicating that the influence of the higher concentration points on the fit has been more evenly distributed.

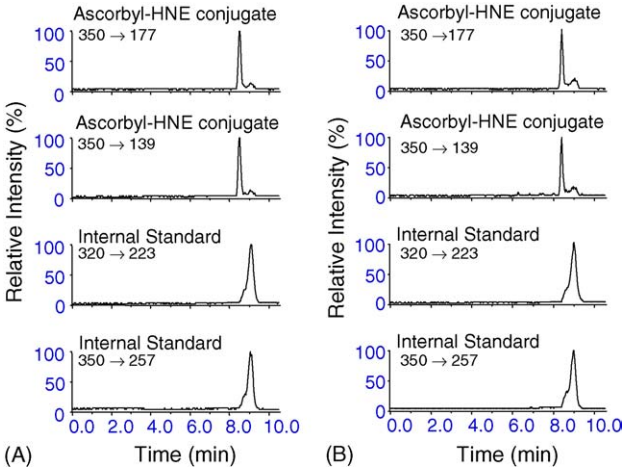

Fig. 5. Chromatograms of the ascorbyl–HNE conjugate and internal standard for nonsmokers (A) and smokers (B). Liquid chromatography/tandem mass spectrometry with multiple reaction monitoring was used for the analysis. The upper two panels' peaks arise from retro-Michael fragmentation of the ascorbyl–HNE adduct (retention time 8.4 min). The peaks in the lower two panels are due to fragmentation of the internal standard, ascorbylated 2-octenal (retention time 8.9 min): m/z 320 $[M + NH_4]^+ \rightarrow m/z$ 257 $[MH - H_2O–CO]^+$ and m/z 223 $[MH - 2H_2O - CO_2]^+$ [40].

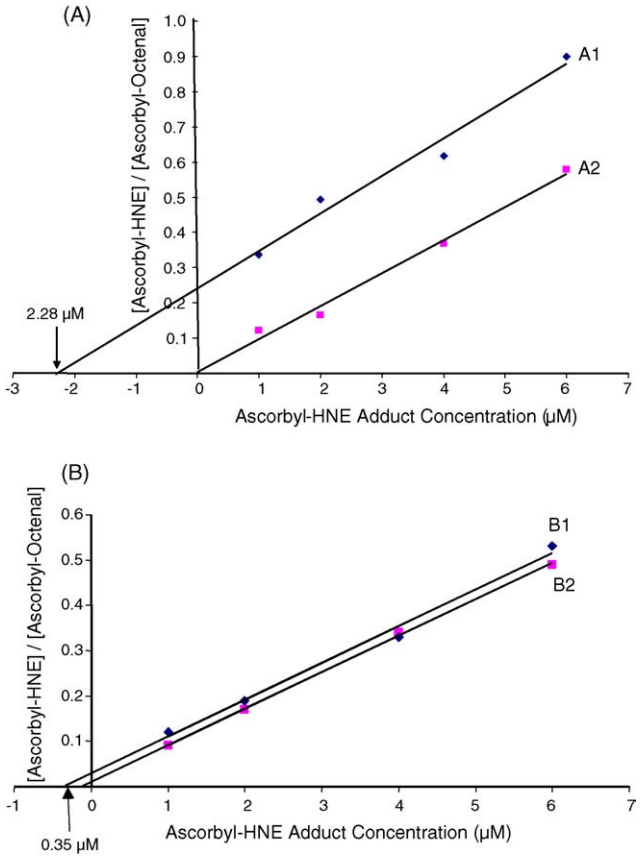

Fig. 6. Standard addition experiments for nonsmokers (A) and smokers (B). Curve A2 and B2 represent external calibration curves derived from analysis of various concentrations of the ascorbyl–HNE adduct with a fixed concentration of internal standard. Curves A1 and B1 were derived from the analysis of aliquots of a plasma sample, either nonsmoker (A) or smoker (B), spiked with various concentrations of synthetic ascorbyl–HNE adduct and a fixed amount of internal standard.

Sample variation due to instrument error and sample preparation was assessed for both groups, nonsmokers and smokers. To test for variation arising from instrumentation, triplicate injections prepared from the same plasma sample were analyzed for the presence of the ascorbyl–HNE conjugate. Using the calibration curve, ascorbyl–HNE conjugate levels were quantitated. Fig. 5 gives examples of chromatograms showing the endogenous ascorbyl–HNE conjugate and the internal standard for both nonsmokers (Fig. 5A) and smokers (Fig. 5B). It was found that the instrument variation was 2.6% R.S.D. for nonsmokers and 3.7% R.S.D. for smokers. Variation arising from sample preparation was examined by analyzing three samples prepared from the same plasma. The total variation due to instrument error and sample preparation was determined to be 3.8% R.S.D. for nonsmokers and 4.0% R.S.D. for smokers.

A problem with current biomarkers of oxidative stress is the formation of ex vivo-artifacts arising from sample instability or sample handling. We have previously confirmed that the presence of the ascorbyl–HNE adduct in plasma is not due to an ex vivo-artifact [40]. However, it was necessary to investigate the stability of the ascorbyl–HNE adduct in order to ensure that its concentration was not fluctuating as a function of time. To this end, aliquots of a plasma sample were analyzed over a time period of one month. The change in the ascorbyl–HNE conjugate concentration was −5.2% (not significant at the $p < 0.05$ level), demonstrating that the conjugate is stable for at least a month at 4 °C.

Standard addition experiments were conducted on nonsmoker and smoker plasma to further assess the accuracy and precision of the method developed for determination of the ascorbyl–HNE conjugate concentration in human plasma (Fig. 6). Aliquots of a nonsmoker plasma sample were spiked with synthetic ascorbyl–HNE adduct (1, 2, 4 and 6 μM) and ascorbyl-octenal adduct (25 μM). The samples were extracted as described previously and analyzed by LC–MS/MS-MRM (Fig. 6A1). Calibration solutions of synthetic ascorbyl–HNE adduct and internal standard were run in parallel (Fig. 6A2). Extrapolation of curve A1 to $y = 0$ gave an ascorbyl–HNE conjugate concentration of 2.28 μM. A blank plasma sample was analyzed using the calibration curve (Fig. 6, curve A2), and the ascorbyl–HNE conjugate concentration was determined to be 2.68 μM. An analogous experiment using smoker plasma was conducted and the results are shown in Fig. 6B. Extrapolation of curve B1 to $y = 0$ gives an ascorbyl–HNE concentration of 0.35 μM. Analysis of this plasma sample using the external calibration curve B2 of Fig. 6 gives a concentration of 0.32 μM. The above results suggest that the developed method is accurate and reproducible.

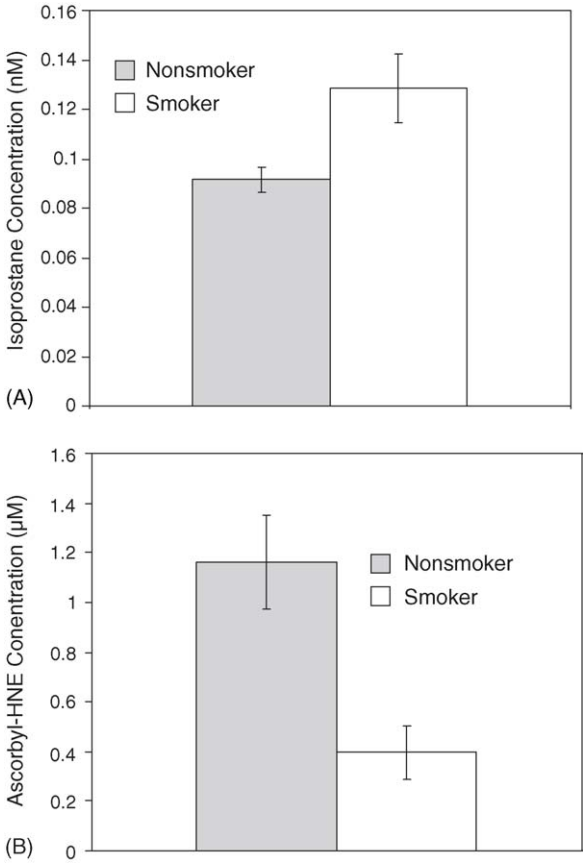

Fig. 7. Plasma levels of $F_{2\alpha}$-isoprostane (A) and the ascorbyl–HNE conjugate (B) in smokers ($n = 10$) and nonsmokers ($n = 10$), presented as mean ± S.E. LC–MS/MS injections were done in duplicate and averaged for all subjects. Results in both panels are statistically significant at the $p < 0.05$ level.

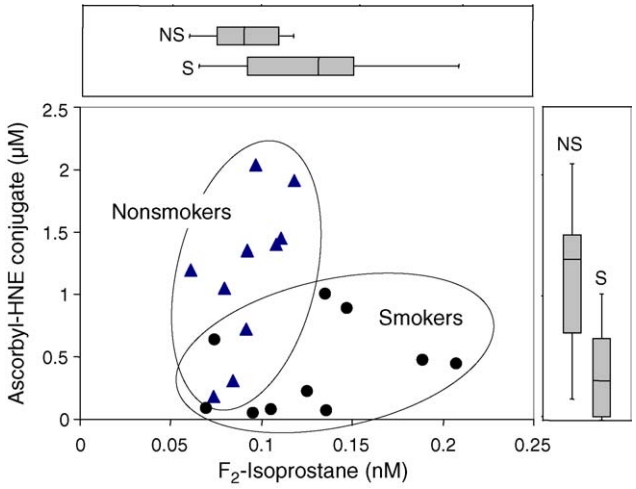

Fig. 8. Scatter and box plots of F_2-isoprostane and ascorbyl–HNE levels in smokers (S, $n = 10$) and nonsmokers (NS, $n = 10$).

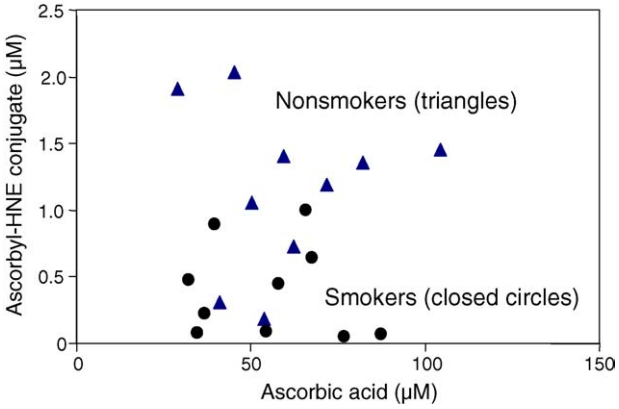

Fig. 9. Scatter plot of ascorbic acid and ascorbyl–HNE conjugate levels in smokers ($n = 10$) and nonsmokers ($n = 10$). Plasma ascorbic acid levels were measured by HPLC with amperometric detection [45].

3.3. Ascorbyl–HNE conjugate as a novel biomarker of oxidative stress

In order to investigate the utility of the ascorbyl–HNE adduct as a novel biomarker of oxidative stress, plasma samples from 20 subjects, 10 smokers and 10 nonsmokers, were analyzed utilizing LC–MS/MS-MRM. F_2-isoprostane levels of the plasma samples were previously reported [42]. On average, the smokers had elevated levels of F_2-isoprostanes and lower plasma ascorbyl–HNE conjugate levels compared to the nonsmokers. Conversely, nonsmokers had higher levels of the ascorbyl–HNE conjugate and lower levels of F_2-isoprostanes (Fig. 7). Correlations between F_2-isoprostane and ascorbyl–HNE conjugate levels within groups were not significant ($p = 0.07$ for nonsmokers and $p = 0.39$ for smokers) (Fig. 8).

It was of interest to determine if a correlation existed between vitamin C levels and ascorbyl–HNE levels. Plasma ascorbate levels of the subjects were previously reported by Bruno et al. [42]. The average ascorbate levels were 51.5 ± 14.8 μM (mean ± S.D.) for nonsmokers and 51.6 ± 19.9 μM for smokers. Fig. 9 illustrates a plot of plasma ascorbic acid concentration vs. the ascorbyl–HNE conjugate concentration. As can be seen in the figure, no significant correlation exists in either group ($p = 0.99$ for nonsmokers and $p = 0.62$ for smokers). This observation demonstrates that the plasma levels of the ascorbyl–HNE conjugate do not merely reflect plasma ascorbate levels.

The observed results are somewhat counterintuitive, since it would be reasonable to expect the concentration of the ascorbyl–HNE conjugate to increase in response to oxidative stress, as F_2-isoprostanes do. Ascorbylation of HNE seems to be compromised in smokers compared to nonsmokers, suggesting that the plasma ascorbyl–HNE levels are a biomarker of the body's physiological response to oxidative stress rather than a direct measure of in vivo lipid peroxidation.

4. Conclusions

Oxidative stress has been linked to a multitude of diseases, including atherosclerosis, Alzheimer's disease and autoim-

mune disorders such as lupus and rheumatoid arthritis. Consequently, tools for the assessment of cellular oxidative stress levels are of interest. Current strategies for assessing oxidative stress levels range from the detection of LPO products, such as F_2-isoprostanes, 4-hydroxy-2-nonenal and malondialdehyde, to monitoring the redox status of antioxidant compounds. While these approaches are useful, chemical instability and artifact formation are potential concerns. Thus, we investigated the feasibility of utilizing a newly-discovered compound, the ascorbyl–HNE conjugate [40], as a novel biomarker of oxidative stress. From an analytical perspective, the ascorbyl–HNE conjugate is not susceptible to ex vivo-artifacts arising from autooxidation, is present at favorable concentrations and is chemically stable. Our data indicate that the ascorbyl–HNE adduct levels in plasma may prove to be useful for the assessment of responsiveness to oxidative stress (and perhaps individual resistance to inflammatory and age-related diseases), which suggests that ascorbylation is a novel detoxification pathway for toxic LPO products.

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