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In Vivo Investigation of Changes in Biomarkers of Oxidative Stress Induced by Plant Food Rich Diets

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It is well established that vegetables and fruit (VF) contain antioxidant phytochemicals. Consequently, it is expected that individuals who consume diets with a high content of VF should be better protected against oxidative cellular damage than individuals who do not, but not all data support this assumption. The objective of this study was to identify possible explanations for this conundrum. The effects of two diets that differed in VF content on markers of oxidative damage were studied. Sixty-four women participated in a 14-day dietary intervention. Subjects consumed on average either 3.6 or 12.1 servings of VF per day. The primary end points assessed were 8-hydroxy-2-deoxyguanosine (8-oxo-dG) in peripheral lymphocyte DNA and 8-isoprostaglandin F-2α (8-iso-PGF2α) excreted in urine. Subjects consuming the high versus low VF diet had lower concentrations of 8-oxo-dG ($p < 0.01$) and of 8-iso-PGF2α ($p < 0.01$). However, the reduction in oxidative end points by high VF was not uniform. Rather, an antioxidant effect was observed primarily in individuals whose oxidative end points at baseline were above the median for the study population. Using change in plasma carotenoids (end point minus baseline measurements) as an index of phytochemical intake, the reduction in oxidative markers was inversely proportional to change in plasma carotenoids; this effect was stronger for lipid peroxidation ($p < 0.01$) than DNA oxidation ($p < 0.05$). These findings imply that increasing exogenous antioxidant exposure may primarily benefit individuals with elevated levels of oxidative stress. Null findings do not necessarily indicate that an antioxidant compound lacks in vivo activity.

KEYWORDS: Antioxidant; fruit; oxidative stress; DNA oxidation; lipid peroxidation; phytochemicals; vegetables

INTRODUCTION

Reactive oxygen species from both endogenous and exogenous sources may be involved in the etiology of diverse human diseases, such as coronary artery disease, diabetes, and cancer (*1*, *2*). Diets rich in fruits and vegetables are associated with a reduced risk for these pathologies, and protection has often been attributed to their phytochemical constituents that have antioxidant activity (*1*, *3*, *4*). However, a small but growing number of reports have failed to demonstrate an effect of increased vegetable and fruit intake on biomarkers of oxidative stress (*5*–*7*). Such reports are puzzling, contrary to biological expectation, and underscore our limited understanding of in vivo factors that regulate redox chemistry (*8*, *9*). This conundrum is particularly problematic given the broad-based interest of the public in antioxidants and health, the increasing number of reports of in vitro studies comparing the antioxidant activities of various foods, and the growing recognition that the results of such tests may have limited meaning in vivo (*9*, *10*).

The intent of the work reported in the present study was to determine whether consumption of an amount of VF similar to that consumed by most Americans (3 to 4 servings per day) or an amount of VF at the upper range of intake recommended (10 or more servings per day) would have differential effects on concentrations of markers of DNA oxidation and lipid peroxidation. These macromolecules were selected because their oxidation has been implicated in the disease processes referenced above (*11*–*14*). We report that in comparison to subjects consuming a low VF diet, the consumption of a high VF diet resulted in lower levels of DNA oxidation and lipid peroxidation. However, the effects observed on oxidative end points by high VF were not uniform. Rather, individuals whose oxidative end points at baseline were above the median for the study population experienced the greatest reduction in oxidative indices.

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Table 1. Botanical Families from Which the Low and High Vegetable and Fruit Diets Were Formulated

botanical family	common fruit and vegetable examples
Actinidiaceae	kiwi
Agaricaceae	mushroom
Chenopodiaceae	spinach, Swiss chard, beet
Compositeae	artichoke, endive, lettuce
Convolvulaceae	sweet potato
Cruciferae	cabbage, broccoli, radish
Cucurbitaceae	cucumber, zucchini, melon
Ericaceae	blueberry, cranberry
Gramineae	corn, bamboo shoots
Leguminosae	chickpeas, lentils, soybeans
Liliaceae	chive, garlic, onion, scallion
Musaceae	banana, plantain
Rosaceae	apple, peach, strawberry
Rutaceae	grapefruit, orange, lemon, lime
Solanaceae	tomato, eggplant, peppers
Umbelliferae	carrot, celery, parsnip, parsley
Vitaceae	grape

MATERIALS AND METHODS

Dietary Approach. To modify VF consumption in a prescribed manner, two diets were developed that provided on average either 3.6 or 12.1 servings of VF per day. The diets were formulated by specifying the amounts and types VF that were to be eaten; VF were selected from 17 botanical families so that a broad range of phytochemicals with potential antioxidant activity were consumed (**Table 1**). Each diet consisted of a fully defined 14-day menu of recipes. Both diets had the same macronutrient content (% kcal from fat, 30%; protein, 18%; carbohydrate, 52%), met the Recommended Dietary Allowances for vitamins and minerals, and were balanced in nutrients from the various food groups. Individuals on the low VF diet consumed on average 1695 kcal/day and those on the high VF diet consumed an average of 1730 kcal/day.

Experimental Design. Recruitment for this study occurred during a meeting to which individuals interested in participating were invited. The purpose of the study was explained, and informed consent was obtained. The duration of the intervention was 14 days. Blood samples (nonfasting) and first void of the morning urine specimens were collected at the time of randomization and again on the morning after the last day of the dietary intervention. A total of 64 individuals completed the intervention: 27 on the low VF diet and 37 individuals on the high VF diet. The age range of the participants was 23−81 years with an average of 48.8 years. On the basis of food logs kept throughout the 14-day intervention, the average intake of VF was 3.6 servings per day for the low VF diet group and 12.1 for the high VF diet group. Pre-intervention body weights were on average 66.2 kg (range 43.4−106.6 kg) and were not significantly affected by either dietary intervention.

Oxidative Markers. *Lymphocyte 8-oxo-dG.* To evaluate the antioxidant activity of the two diets, the concentration of 8-oxo-dG in DNA isolated from peripheral lymphocytes was determined. 8-oxo-dG is a relatively abundant and readily detected product of oxidative DNA damage and as such is regarded as a useful and relevant marker for cellular oxidative stress (*13*). The decision to assess oxidative damage in DNA isolated from lymphocytes was made for several reasons. Lymphocytes are relatively easy to obtain, their half-life in blood is short, and they provide a general estimate of the oxidative stress, at the genomic level, to which an organism is subjected (*15*, *16*). Blood (nonfasting) for lymphocyte isolation was processed in Becton Dickinson Cell Preparation tubes that contained sodium citrate as the anticoagulant. Lymphocytes were harvested from these tubes and were frozen at −80 °C in phosphate-buffered saline containing 10% dimethyl sulfoxide for subsequent isolation of DNA from nuclei. Nuclei were isolated from lymphocytes by use of a nonionic detergent, and DNA was isolated from nuclei by a method employing proteinase K and organic extraction. 8-oxo-dG and dG in DNA from lymphocytes were measured by use of reverse-phase HPLC with electrochemical and spectrophotometric detection for 8-oxo-dG and dG, respectively. The analysis of 8-oxo-dG was performed with vigilant attention to conditions that can induce the artificial formation of 8-oxo-dG, as discussed in a recent communication from our laboratory (*17*). Analysis was performed on identical samples processed in duplicate.

Urinary 8-iso-PGF2α. Analysis of 8-iso-PGF2α in urine provides a time-averaged index of lipid peroxidation over a relatively prolonged interval that offers greater utility than the more transitory information provided by analysis of blood. Moreover, urine contains very little arachidonic acid, thereby limiting artifactual oxidation and isoprostane production after sample collection. Since we have found intraindividual day-to-day excretion of urinary 8-iso-PGF2α to vary considerably in human subjects, we elected to measure 8-iso-PGF2α abundance in pooled urine samples generated from multiple specimens. First void of the morning urine was collected without preservative in plastic vessels on three consecutive days. The decision to use first voids rather than 24-hour collections was based on data indicating that 24-hour averages expressed per mg creatinine were not statistically different from values obtained from first voids and on our experience that collecting reliable 24-hour urine samples from free living subjects is problematic.

As noted in ref (*18*), early studies of isoprostanes were limited in scope by the technical demands of sample processing for gas chromatography−mass spectrometry. However, as reviewed in ref (*19*), reliable ELISA assays, such as that reported in ref (*20*), for specific isoprostanes, have become available. These assays give results that parallel those obtained via GC-MS. In this study, urine samples from each three consecutive day collection period were pooled. Urine samples were diluted 10-fold with Tris buffered saline to standardize pH and ionic strength. Diluted samples were then analyzed for 8-iso-PGF2α concentration according to the manufacturer's directions using an ELISA kit from Assay Designs (Ann Arbor, MI). All time points from a subject were included within an analytical run, and both treatment groups were represented approximately equally within a run as well. This approach minimized the chances of having interassay variability masquerade or obscure treatment effects. Identical samples of control urine were extracted and analyzed with every run to monitor assay performance. To keep analysts blind to treatment group, the project biostatistician provided pairs of subject accession numbers such that each pair contained one subject from each treatment group, and analytical runs were configured from those pairs. 8-iso-PGF2α values were expressed per mg creatinine. Creatinine concentration was measured using a kit (Cat#558-A, Sigma-Aldrich, St Louis, MO) on the basis of visible absorbance of the Janovski complex chromophore generated by derivitization with picrate. The colorimetric creatinine assay is very consistent; interassay precision of identical control urine samples included with every analysis was 2.4% for 32 analytical runs over 58 days.

Plasma Carotenoids. The concentration in plasma of five carotenoids, alpha-carotene, beta-carotene, lycopene, lutein, and beta-cryptoxanthine, was determined to provide a biochemical index of compliance to the intervention diets. Plasma was separated from nonfasting blood, collected using potassium EDTA as an anticoagulant, and stored at −80 °C for subsequent measurement of carotenoids. Carotenoid concentrations were determined by use of a reverse-phase HPLC method on the basis of those of Peng (*21*) and Hess (*22*). Briefly, plasma was extracted with hexane, which was removed under reduced pressure. The extract was reconstituted with mobile phase and was separated by isocratic reverse-phase chromatography. Photodiode array detection facilitated quantitation of five analytes at three different wavelengths from a single injection and enabled use of spectral data for confirmation of peak identity. Standard curves for each analyte generated with each analytical run proved to be remarkably stable over time. Working standard solutions containing all five analytes were prepared from a stock carotenoid solution whose concentration was regularly monitored by use of absorbance photometry. Stock solutions of carotenoids in hexane and chloroform were stable at −20 °C when preserved with butylated hydroxytoluene. A human plasma control sample was extracted and analyzed with each run to monitor assay precision. Analyses were configured such that all time points from a subject were analyzed together, and each run included approximately

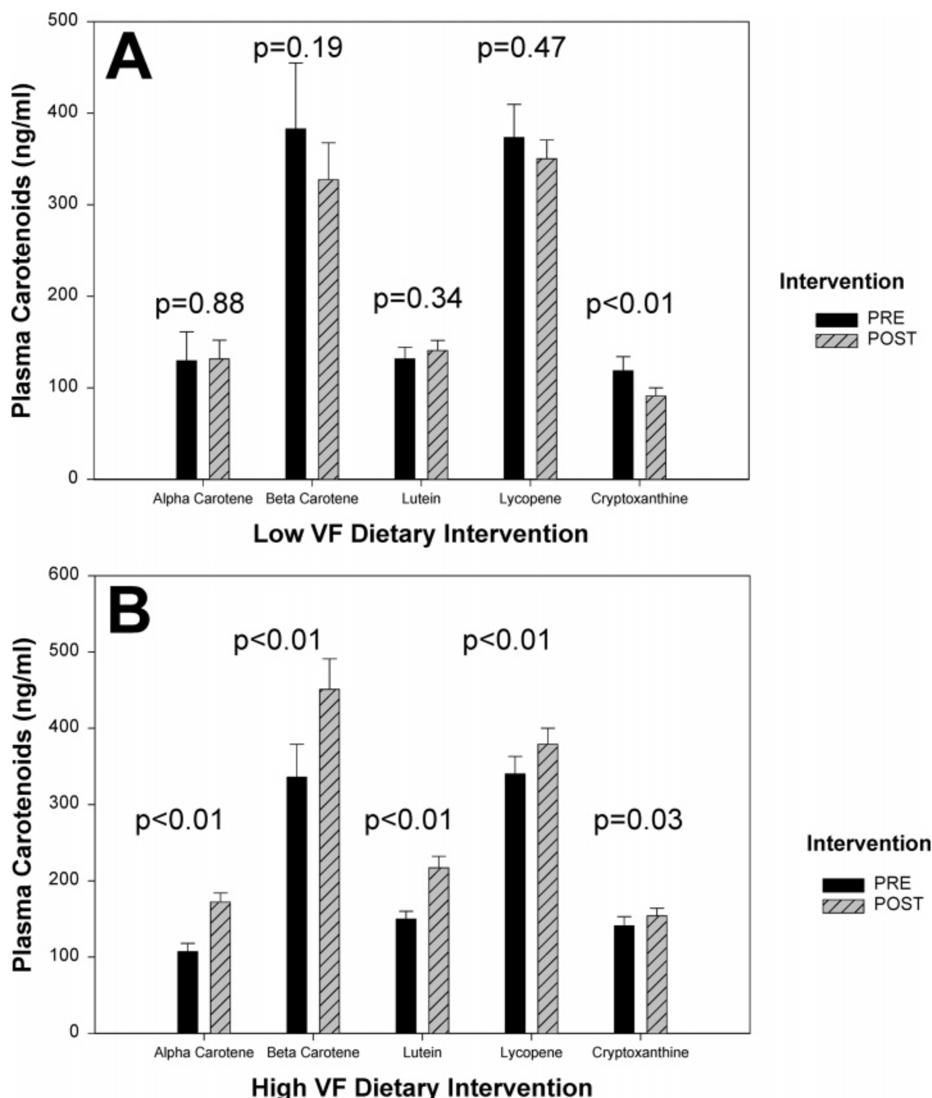

Figure 1. Effects of a low VF dietary intervention (panel A) and a high VF dietary intervention (panel B) on pre- and post-intervention levels of plasma carotenoids. Values are means ± SEM. *p*-values shown are for the comparison of pre- versus post-intervention levels of each carotenoid.

equal numbers of subjects from both treatment groups. Analysts were blind to treatment group.

Statistical Analyses. Tests for the overall change (pre-intervention versus post-intervention values) in the outcomes, urinary 8-iso-PGF2α and lymphocyte 8-oxo-dG, were performed using repeated measures, mixed effects model (*23*). Changes in other parameters were assessed using a paired t-test (*24*). All statistical tests were two-tailed.

RESULTS

Plasma Carotenoids. The effects of the dietary interventions on plasma levels of five commonly reported carotenoids, α- and β-carotene, lutein, lycopene, and β-cryptoxanthin, are shown in **Figure 1**. In the low VF diet group, post-intervention plasma carotenoid levels either remained essentially unchanged or decreased numerically in comparison to pre-intervention levels; however, only the decrease in β-cryptoxanthin, which was 23.3% lower in the post-intervention samples, was statistically significant ($p < 0.01$). In the high VF diet group, the post intervention plasma carotenoid concentrations were elevated in all cases in comparison to pre-intervention values but to different degrees. All differences were statically significant (*p*-values ranged from $p < 0.01$ to $p = 0.03$).

Lymphocyte 8-oxo-dG. The high VF intervention resulted in a 16.5% reduction in lymphocyte 8-oxo-dG (pre- vs post-intervention, $p < 0.01$), whereas no reduction was observed in the low VF intervention group (pre- vs post-intervention, 0.5%, $p = 0.77$, **Figure 2**). When the difference in pre- versus post-intervention levels of 8-oxo-dG observed in the low VF diet group was subtracted from that observed in the high VF diet group, the effect of the high VF dietary intervention in reducing 8-oxo-dG remained statistically significant ($p = 0.04$).

Urinary 8-iso-PGF2α. The effect of the dietary interventions on lipid peroxidation measured as 8-iso-PGF2α in urine is shown in **Figure 3**. The urinary concentration of 8-iso-PGF2α was numerically lower in the post-intervention versus the pre-intervention samples of subjects in both intervention groups. The extent of reduction was greatest in the high VF intervention group (30.7%, $p < 0.01$); the 10.9% reduction observed in the low VF intervention group did not reach the level of statistical significance ($p = 0.09$). The difference between the change in pre- versus post-intervention urinary levels of 8-iso-PGF2α observed in the low VF diet group and the change in pre- versus post-intervention urinary levels of 8-iso-PGF2α observed in the high VF diet group was statistically significant ($p = 0.04$).

Relationships among Change in Plasma Total Carotenoids and Change in Oxidative Markers. To evaluate the idea that individuals who had the greatest increases in vegetable and fruit intake from their baseline levels of consumption experienced

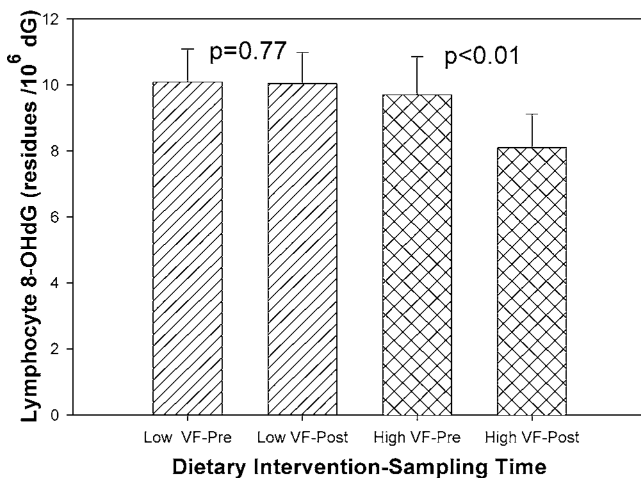

Figure 2. Effect of a low and a high VF dietary intervention on pre- versus post-intervention levels of lymphocyte 8-oxo-dG. *p*-values shown are for the comparison of pre- versus post-intervention levels of 8-oxo-dG for each dietary intervention.

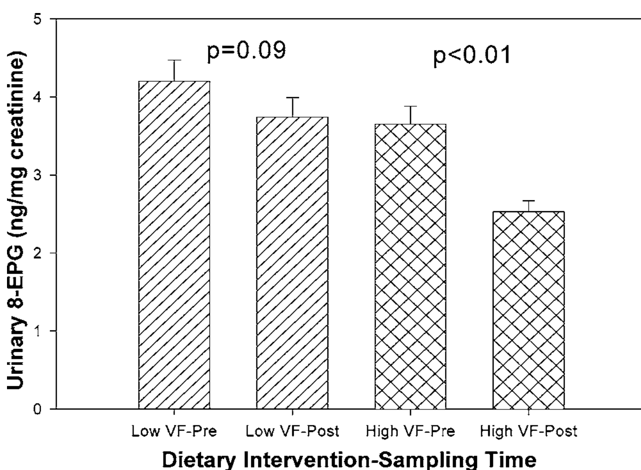

Figure 3. Effect of a low and a high VF dietary intervention on pre- versus post-intervention levels of urinary 8-iso-PGF2α. *p*-values shown are for the comparison of pre- versus post-intervention levels of 8-iso-PGF2α for each dietary intervention.

the greatest reductions in oxidative indices, each individual's change in total plasma carotenoids, lymphocyte 8-oxo-dG, and urinary 8-iso-PGF2α (post minus pre-intervention values) was computed. Subjects were then grouped into tertiles by the magnitude of change in plasma carotenoids; this was done for all individuals irrespective of the intervention in which they participated. The mean change in lymphocyte 8-oxo-dG and urinary 8-iso-PGF2α for each tertile was then computed. The results of these analyses are shown in **Figure 4**. Mean changes in plasma carotenoids for lowest, intermediate, and highest tertile were −0.7%, 48%, and 196%, respectively. As shown in **Figure 4**, panels A and B, the reduction in oxidative markers was inversely proportional to change in plasma carotenoids; this effect was stronger for lipid peroxidation ($p < 0.01$) than for DNA oxidation ($p < 0.05$).

Response to Dietary Interventions Based on Pre-Intervention Oxidative Status. To test the hypothesis that the response to the dietary interventions was not uniform but rather depended on pre-intervention (baseline) oxidation status, groups were subdivided into individuals who were below or above the median value for each oxidative marker at baseline. The effects of both dietary interventions on concentrations of 8-oxo-dG and 8-iso-PGF2α are shown in **Table 2**. Concentrations of 8-oxo-dG were

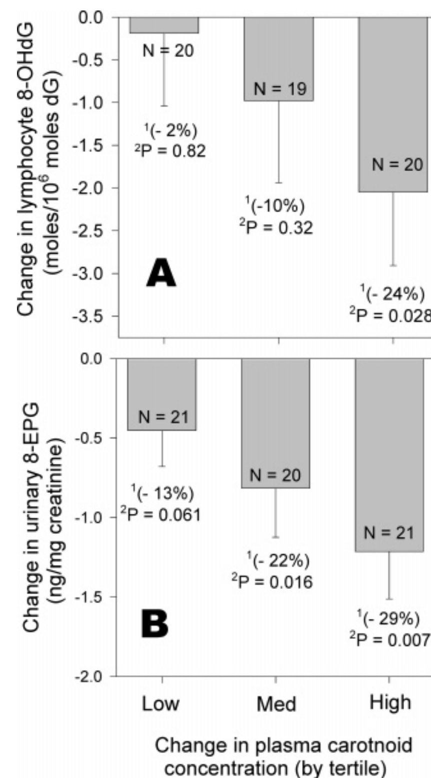

Figure 4. The change in lymphocyte 8-oxo-dG (panel A) and urinary 8-iso-PGF2α (panel B) expressed as a function of change in plasma total carotenoid concentration. [1]Percent difference between mean values for each oxidative index (post- versus pre-intervention) in each of the three tertiles of change in plasma carotenoids. [2]*P*-values from paired, two-tailed *t*-test performed on oxidative index values (post- versus pre-intervention) in each of the three tertiles.

unaffected by either dietary intervention in individuals whose baseline 8-oxo-dG was below the median (8.1 residues 8-oxo-dG/10^6dG), whereas the concentration of this analyte was reduced in both interventions in individuals who entered the study above the median. However, the reduction was statistically significant only in the high VF group. A pattern similar to that observed for DNA oxidation was also observed for the changes in concentrations of urinary 8-iso-PGF2α (median value = 3.68 ng/mg creatinine). The effects of both dietary interventions were greatest in individuals who entered the study above the median. The high VF intervention also reduced levels of lipid peroxidation in individuals who were below the median at baseline, but the magnitude of the effect was markedly attenuated relative to that observed in individuals who entered the study above the median.

DISCUSSION

A whole foods approach was used for hypothesis testing in this study. Adherence to both the low VF and high VF dietary regimes was greater than 90% on the basis of the evaluation of food record data maintained by participants throughout the study. Thus, the intent of this study, unlike other approaches that have focused on a specific food, food extract, or chemical constituents, was to determine if increasing overall phytochemical exposure in the context of a nutritionally balanced diet that met national dietary guidelines would exert in vivo antioxidant activity and if the response was uniform across the study population.

Carotenoids are widely distributed in VF, and while the amount and types of carotenoids differ among various plant

Table 2. Effect of the Low and High Vegetable and Fruit Diets on the Concentrations of 8-oxo-dG in Lymphocyte DNA and on 8-iso-PGF2α in Urine on the Basis of Baseline Values of Each Oxidative Index

baseline concentration	dietary intervention	pre-intervention[a]	post-intervention	mean difference	*p*-value[b]
		8-oxo-dG residues/10^6dG			
below median	low VF	6.6 ± 0.3	7.5 ± 0.5	0.9	0.18
	high VF	5.1 ± 0.4	5.3 ± 0.4	0.2	0.75
above median	low VF	13.4 ± 1.4	12.4 ± 1.5	−1.0	0.13
	high VF	15.1 ± 1.7	11.4 ± 1.9	−3.7	0.02
		8-iso-PGF2α ng/mg creatinine			
below median	low VF	3.13 ± 0.21	3.21 ± 0.47	0.08	0.86
	high VF	2.66 ± 0.12	2.25 ± 0.13	−0.41	0.01
above median	low VF	4.94 ± 0.32	4.10 ± 0.25	−0.84	0.01
	high VF	5.03 ± 0.26	2.92 ± 0.24	−2.11	0.001

[a] Values are means ± SEM. Median values: 8-oxo-dG, 8.1 residues/10^6dG; 8-iso-PGF2α, 3.68 ng/mg creatinine. [b] *p*-values from paired, two-tail *t*-test performed on oxidative index values (post- versus pre-intervention).

foods, overall intake of VF is highly correlated with plasma carotenoid concentrations. The data on the effects of the two VF diets on plasma carotenoid concentrations shown in **Figure 1** provide convincing evidence that plant food intake was markedly increased by the high VF diet in comparison to the low VF diet. That the levels of carotenoids of each type reported were significantly increased also reflected the broad botanical diversity of the high VF diet and supports the notion that exposure to a wide range of chemical antioxidants was tested in this study.

While considerable evidence indicates that many of the chemical constituents of VF have antioxidant activity in vitro, little is known about whether ingesting different amounts of these chemicals via consuming a nutritionally balanced diet with high VF content alters oxidative damage to cellular macromolecules in vivo. As shown in **Figure 2**, the concentration of a DNA oxidation product, 8-oxo-dG, was reduced 16.5% in lymphocyte DNA during the 2-week high VF dietary intervention, while it was essentially unchanged in subjects assigned to a low VF diet intervention (0.5% reduction, $p = 0.77$). To examine whether the difference in these responses could be attributed to increasing VF consumption, the effect of the high VF diet was adjusted for that observed in the low VF diet, that is, the intervention adjusted difference. The results of this analysis showed that the effects of the high VF diet were statistically significant ($p = 0.04$), a finding that supports the hypothesis that increasing VF consumption per se, rather than the process of participating in the dietary intervention, reduced lymphocyte levels of 8-oxo-dG. Assuming that levels of DNA oxidation in lymphocytes reflect the equilibrium between the rates of DNA damage and repair, this implies that the oxidative burden of the host, as reflected in lymphocyte DNA, was modestly reduced by increasing VF consumption.

Another end point used to assess the effects of the VF dietary interventions on in vivo oxidative damage was lipid peroxidation measured in urine as 8-iso-PGF2α. The data reported in **Figure 3** show that urinary 8-iso-PGF2α was reduced to a greater extent in individuals consuming the high VF diet (30.7%, $p < 0.01$) relative to those on the low VF diet (10.9%, $p = 0.09$). Further analysis of these data in which the intervention adjusted difference was determined supported the hypothesis that increasing VF consumption decreased lipid peroxidation ($p = 0.04$). These findings parallel those reported for lymphocyte 8-oxo-dG and are consistent with reports that link cellular events resulting in lipid peroxidation to those associated with the oxidation of DNA (*12*, *25*).

On the basis of these findings, we ranked all individuals in the study by the change in total plasma carotenoids that they experienced during the dietary intervention. These values were then used to divide individuals into tertiles of change in plasma carotenoids and by implication VF intake. We also computed the change in lymphocyte 8-oxodG and 8-iso-PGF2α for each individual. Our hypothesis was that those individuals who experienced the greatest increases in plasma carotenoids would also have experienced the greatest reductions in markers of oxidative damage. The data shown in **Figure 4** support this hypothesis and provide further support that increased VF intake reduced cellular oxidation by reactive oxygen species (ROS).

Since the existence of ROS was established in 1953, the awareness of the biological effects of ROS has grown with the realization that ROS are involved not only in the pathophysiology of many chronic diseases but also in the regulation of numerous cellular processes and that they are formed continuously as a part of normal cellular metabolism (*1*, *2*). Thus, not surprisingly, as for most chemical species in biology, the poison is in the dose. Low concentrations of ROS play an essential role in normal biological function; only when their formation or disposal is misregulated do ROS exert deleterious effects. This distinction between physiological and pathological effects is frequently viewed in terms of oxidant stress and antioxidant activity. Both endogenous and exogenous chemicals can contribute to the pools of oxidants and antioxidant present in cellular systems. An issue that has received limited attention relates to the in vivo conditions under which it should be anticipated that dietary chemicals with antioxidant activities would exert detectable antioxidant effects. To explore this issue, a post hoc analysis was performed on the data, and the results are shown in **Table 1**. The hypothesis tested was that some level of oxidative damage should be expected as a normal product of physiological levels of ROS and that exogenous antioxidants would not be expected to influence oxidative damage under these conditions. Rather, exogenous antioxidants would be expected to have the greatest impact in individuals whose levels of oxidation are elevated. We operationally defined oxidative status as being elevated in individuals whose level of lymphocyte 8-oxo-dG or 8-iso-PGF2α was above the median of the study population for each oxidative marker. The data in **Table 1** indicate that both dietary interventions had their primary effects on oxidative markers in individuals whose oxidation status was elevated; the effects of the high VF diet were statistically significant and the magnitude of the effects appeared greater for lipid peroxidation than for DNA oxidation. While there are many implications of these findings that merit further investigation, for example, why did the low VF diet also reduce lipid peroxidation, in our judgment, one implication that is critical to underscore is that

increasing the ingestion of antioxidant phytochemicals may not always result in decreases in markers of oxidative cellular damage in vivo. Rather, it appears that in vivo antioxidant effects of exogenous chemicals are conditional depending on the oxidative stress burden of an individual. Thus, in vivo tests of the antioxidant activity of various phytochemicals are likely to be most sensitive when conducted with oxidatively stressed individuals; urinary evaluation of whole body lipid peroxidation may provide a noninvasive approach for screening individuals who are most likely to respond to an antioxidant intervention.

In summary, the results of this study addressed the antioxidant conundrum as outlined in the Introduction and as summarized in ref (*9*). Diets with high levels of VF did exert in vivo antioxidant activity in terms of reducing concentrations of a DNA oxidation product measured in lymphocytes and a product of lipid peroxidation assayed in urine. However, the fact that the reduction in oxidative damage was observed primarily in individuals with increased levels of either oxidation product at the study baseline is consistent with the idea that dietary antioxidants can benefit individuals in whom endogenous cellular antioxidant mechanisms have failed to maintain oxidation at levels that are considered physiological. The extent to which individuals experiencing nonphysiological levels of oxidation are included in studies of the antioxidant activity of dietary chemicals may explain, in part, the variability in published results and the emergence of the antioxidant conundrum. Further studies are needed in human populations to better define levels of oxidation that are judged to be physiological versus those considered to have pathophysiological significance.

ABBREVIATIONS USED

8-iso-PGF2α, 8-isoprostaglandin F-2α; 8-oxo-dG, 8-hydroxy-2-deoxyguanosine, dG, deoxyguanosine; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetracetic acid; ELISA, enzyme-linked immunosorbent assay; GC-MS, gas chromatography−mass spectrometry; ROS, reactive oxygen species; SPE, solid-phase extraction; VF, vegetable and fruit.

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