Exhaled Ethane and Antioxidant Vitamin Supplements in Active Smokers*

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To determine the effect of nutritional agents on lipid peroxidation, 10 smokers were given 6 mg beta carotene, 200 IU vitamin E, and 250 mg vitamin C 4 times daily for 3 weeks. Lipid peroxidation was assessed by measuring baseline and postsupplementation levels of exhaled ethane. There was a 29% decrease in mean (±SD) exhaled ethane (4.06 ± 1.49 vs 2.90 ± 1.29 pmol·kg⁻¹·min⁻¹), with individual levels decreasing in 8 of the 10 smokers (p<0.05, Wilcoxon sign rank test). Three nonsmokers had very low baseline levels of ethane that did not change with supplementation. Ethane production correlated with active (packs per day) and lifelong (pack-years) tobacco consumption. Also, a strong correlation was found between the decline in ethane output after micronutrient supplementation and the presupplement FEV₁. Therefore, antioxidant vitamin supplementation resulted in attenuation of smoking-related lipid peroxidation, and the decreases in ethane production appears to be associated with preserved lung function.

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Key words: ascorbic acid; carotene; ethane; lipid peroxidation; smoking; vitamin E

Free radical activity has been implicated in the pathogenesis of a variety of illnesses, and cigarette smoke is a direct and indirect source of large numbers of these biotoxic agents.¹⁻⁵ Not only does cigarette smoke contain 10¹⁵ free radicals per gram of tar, but smoke also induces the release of reactive oxygen species by inflammatory cells into the lung. In theory, these free radicals may play a role in carcinogenesis through oxidation of DNA⁶⁻⁷ and in emphysema through inactivation of human antiproteases.⁸⁻¹⁰ They also initiate and propagate peroxidation of phospholipids in cellular membranes, leading directly to pathologic alterations in membrane structure and function and to the production of biotoxic compounds.¹¹

Therefore, attenuation of free radical activity by antioxidant nutrients may be applicable in disease prevention. Beta-carotene quenches singlet oxygen and reacts with the peroxyl radicals of the lipid peroxidation cascade.¹³ Vitamin C reacts directly with superoxide anions, hydroxyl radicals, and peroxyl radicals in aqueous solutions, and maintains vitamin E in the reduced state.¹³⁻¹⁵ Vitamin E, the most effective chain-breaking membrane-associated antioxidant, competes for peroxyl radicals at a faster rate than polyunsaturated fatty acids, protecting polyunsaturated fatty acids against oxidation.¹¹,¹⁶

An association of these nutrient antioxidants with disease prevention has been observed in epidemiologic studies.¹⁷ For example, cancer mortality has been associated with low serum levels of beta carotene and vitamin E.¹⁸,¹⁹ There is an inverse relationship between vitamin A activity and carotene intake and the incidence of bronchus cancer.²⁰,²¹ Beta carotene and vitamin E probably have a role in preventing oral cavity cancer, another smoking-related disease.²² Furthermore, increased dietary intake of antioxidant vitamins has been associated epidemiologically with higher values of FEV₁.²³ Consequently, observations that smoking reduces plasma levels of beta carotene and vitamin C²⁴,²⁵ and that vitamin E levels are reduced in adipose tissue²⁶ and alveolar fluid of smokers when compared with nonsmokers²⁷ may have pathophysiologic significance.

A variety of methods have been applied to assess free radical activity or damage, including the measurement of exhaled ethane. This gas, a byproduct of lipid peroxidation of ω-3 fatty acids (eg, linolenic acid), has recently been shown by us to be elevated in the exhalate of cigarette smokers as compared with nonsmokers.²⁸ We report herein a pilot study that suggests that antioxidants reduce lipid peroxidative activity in smokers. Secondarily, we also examined associations between exhaled ethane, tobacco use, and spirometric indexes of lung function.
Table 1—Population Characteristics*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>52.5±15</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>9/1</td>
</tr>
<tr>
<td>Cigarette use, pack-yr</td>
<td>51.5±11.4</td>
</tr>
<tr>
<td>Lung function</td>
<td></td>
</tr>
<tr>
<td>FEV/FVC</td>
<td>68.4±3.0</td>
</tr>
<tr>
<td>FEV₁ (FEV₁ % predicted)</td>
<td>3.03±0.3 L (89.1±5.8)</td>
</tr>
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</table>

*Expressed as means±SD.

Materials and Methods

Subjects

Twelve current smokers were recruited from staff and patients at the Tucson Veterans Affairs Medical Center. One subject prematurely discontinued the study and another was excluded because he was already taking vitamin supplements. Thus, ten smokers completed the study. None had active illnesses or had taken vitamin supplements for at least 2 months prior to enrollment. Three nonsmoking control subjects were also included. All subjects gave written informed consent prior to participation in the study.

Protocol

After demographic information was obtained, spirometry was performed to access lung function, using the equations of Morris et al.25 to determine percent predicted values. On initiation of the study and at the end of the 3-week trial, exhalate and antecubital venous blood were collected to measure breath ethane and plasma nutrient levels. Subjects were requested not to eat or smoke for at least 3 h prior to ethane measurements, since we have previously shown that exhaled ethane falls to a plateau after that interval.25 After baseline samples were obtained, subjects were given tablets consisting of 6 mg beta carotene, 250 mg vitamin C, and 200 IU vitamin E (d-alpha-tocopherol) to be taken 4 times daily for 3 weeks. Subjects were asked to maintain their usual diets and cigarette consumption during the course of the study. On completion of the trial, compliance was determined by pill count.

Venous blood was collected in foil-wrapped heparinized tubes for measurements of plasma beta carotene and vitamin E levels. Tubes were placed on ice, centrifuged at 1,000xg for 10 min, and the plasma stored at −80°C till analysis. Blood samples were fasting, morning specimens.

Analyses and Measurements

Breath ethane analyses were performed according to previously reported methods,28 with minor modifications. Subjects breathed hydrocarbon free air (Ultra Zero Air; Liquid Air, Tucson, Ariz) through a one-way valve for at least 2 min to wash any contaminating hydrocarbons from their lungs. The length of time during which the washout period was performed depended on spirometry: patients whose FEV₁ percent predicted was less than 50% were washed out for 4 min, those with FEV₁ percent predicted of less than 90% washed out for 3 min, and those above 90% washed out for 2 min. All subjects took several vital capacity breaths during the washout period. This exhalate was discarded. Next, 2 additional minutes of volume of exhalate was collected into a five-layered Douglas bag. Using a vacuum attached to rotameters, duplicate 6-L samples of exhalate were evacuated at 200 mL/min for 30 min over cold traps of activated charcoal housed in ethylene glycol/water (1:1 v/v) slurry frozen at −80°C. The volume of exhalate remaining in the bag was quantified with a spirometer (Collins Pulmonary Testing System; Braintree, Mass), allowing determination of exhaled volume and minute ventilation. The charcoal containing the adsorbed gases was poured into a test tube of known volume and sealed with an open screw top housing a Teflon septum. The tube and its contents were heated for 3 min at 250°C to liberate the adsorbed hydrocarbons. Five milliliters of the headspace gas was removed in a calibrated precision sampling syringe and injected into a gas chromatograph (Hewlett-Packard 5890; Palo Alto, Calif), containing a 2-m glass column packed with Carbowax 60/80 (Alltech; Deerfield, Ill.) maintained isothermal at 220°C. The ethane retention time was 4.74 min. The ethane content of the hydrocarbon-free inspirate was also determined daily and was subtracted from the raw ethane measurements of the subjects. The final breath ethane output levels were expressed as pmoles·min⁻¹·kg⁻¹. Plasma beta carotene and vitamin E levels were measured by photodiode array detector monitoring of high-pressure liquid chromatography effluent, which has previously been detailed by Peng and colleagues.26 Because of rapid changes in value, vitamin C levels were not measured.

Statistical Evaluation

Plasma vitamin and exhaled ethane levels were compared by Student’s t test analysis for paired variables (two tailed). Continuous group data were assessed by linear regression analysis. Statistical significance was assumed for p<0.05.

Results

Population Description/Protocol Compliance

The characteristics of the study population are shown in Table 1. Body weight and dietary habits did not change significantly over the 3-week trial period. Overall compliance (by pill count) was 92%. After supplementation, beta carotene levels in smokers rose from 9.1±6.1 (SD) to 102.7±61.8 μg/dL (p<0.001), and vitamin E levels rose from 0.988±0.251 to 2.747±0.850 mg/dL (p<0.001). Individual values are shown in Table 2. A linear correlation was present for the changes in beta carotene and vitamin E (r=0.680, p<0.05). In the nonsmokers, beta carotene and vitamin E levels also rose significantly after supplementation (12.3±5.30 to 122.3±32.3 μg/dL beta carotene [p<0.02], and 1.021±0.257 to 2.958±0.487 mg/dL vitamin E [p<0.01]).

Ethane Levels and Antioxidants

Exhaled breath ethane values are presented in Table 2. Levels fell in 8 of the 10 subjects and increased in only 2 subjects after antioxidant supplementation (p<0.05, Wilcoxon sign rank test). There was also a decline in mean ethane production before (4.06±1.49 [SD] pmol·kg⁻¹·min⁻¹) and after (2.90±1.29 pmol·kg⁻¹·min⁻¹) antioxidant supplements. In the 3 nonsmokers, baseline ethane levels were low and did not change after vitamin supplementation (0.36±0.09 vs 0.18±0.23 pmol·kg⁻¹·min⁻¹, p=NS). As expected, these levels were significantly lower than the corresponding values for smokers before (p<0.05) and after (p<0.02) vitamin supplementation, confirming our previously reported results.28

There were no correlations between ethane levels and levels of either of the two measured antioxidants,
nor were there correlations between the changes in antioxidant levels and exhaled ethane.

**Ethane and Smoking**

Although there was an expected linear relationship between age and pack-years (r=0.696, p<0.05), we found no correlation between age and ethane production in smokers (r=0.451, p=NS). The influence of smoking history (measured in packs per day or pack-years) on exhaled ethane was also assessed (Fig 1). Although there was no correlation between the baseline exhaled ethane and packs per day (r=0.318, p=NS), there was a significant correlation between the postantioxidant levels of ethane and packs per day (r=0.671, p<0.05). Similarly, pack-years were not correlated with preantioxidant ethane levels (r=0.557, p=NS), but were correlated with postantioxidant ethane levels (r=0.756, p<0.02).

**Ethane and Lung Function**

No correlation was found between absolute values for FEV₁ and ethane, either before or after vitamin supplementation. However, there was a strong inverse correlation between antioxidant-induced decreases in exhaled ethane and FEV₁ (percent predicted) in smokers (r=0.926, p<0.001) (Fig 2).

**DISCUSSION**

Petruzzelli and colleagues31 have demonstrated higher levels of thiobarbituric acid-reactive substances in lung parenchyma from smokers vs nonsmokers, indicating pulmonary lipid peroxidation. Others have identified elevated thiobarbituric acid-reactive substances in the plasma and serum of smokers.32 Our previous observation that smokers exhale more ethane than nonsmokers was confirmed in this study, suggesting that peroxidation induced by smoking can be measured noninvasively.28 Other investigators have recently reported similar findings, eg, Schwarz and colleagues33 found increased ethane production in smoking than in nonsmoking pregnant women. The present study supports the contention that nutrient antioxidants attenuate this peroxidation, although 3 weeks of supplementation was not sufficient to decrease ethane production to the level of nonsmokers. Being a pilot investigation, the small sample size

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**Table 2—Vitamin Levels and Exhaled Ethane Before and After Supplementation**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Beta Carotene, μg/dL</th>
<th>Vitamin E, mg/dL</th>
<th>Ethane, pmol·kg⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>12.6</td>
<td>1.374</td>
</tr>
<tr>
<td>2</td>
<td>9.2</td>
<td>129.8</td>
<td>1.056</td>
</tr>
<tr>
<td>3</td>
<td>2.6</td>
<td>45.3</td>
<td>0.709</td>
</tr>
<tr>
<td>4</td>
<td>10.3</td>
<td>103.1</td>
<td>1.019</td>
</tr>
<tr>
<td>5</td>
<td>7.4</td>
<td>66.6</td>
<td>0.881</td>
</tr>
<tr>
<td>6</td>
<td>12.8</td>
<td>185.5</td>
<td>1.230</td>
</tr>
<tr>
<td>7</td>
<td>2.4</td>
<td>23.7</td>
<td>0.621</td>
</tr>
<tr>
<td>8</td>
<td>17.9</td>
<td>154.7</td>
<td>1.276</td>
</tr>
<tr>
<td>9</td>
<td>8.6</td>
<td>157.7</td>
<td>0.872</td>
</tr>
<tr>
<td>10</td>
<td>18.3</td>
<td>150.7</td>
<td>0.934</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11.1</td>
<td>123.7</td>
<td>0.928</td>
</tr>
<tr>
<td>2</td>
<td>16.6</td>
<td>153.9</td>
<td>0.792</td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
<td>89.4</td>
<td>1.343</td>
</tr>
</tbody>
</table>

**Figure 1.** Relationship between smoking habits and ethane production. Depicted are the correlations between pack per day current tobacco use and exhaled ethane before (top left) and after (top right) vitamin supplementation, and for pack-years and ethane before (bottom left) and after (bottom right) supplementation.
poses limitations to our study, which will be the subject of future trials. All three nutrients were given, which may not be necessary. Their lack of toxicity, and availability in a single, combined capsule form for convenient dosing, led us to the dose schedule used herein. Larger follow-up trials, using double-blind, placebo-controlled, crossover designs are planned to confirm these findings and assess whether single agents are as active as combinations.

It is disputed whether ethane or pentane more accurately indicates lipid peroxidation in vivo. Breath ethane is derived from the peroxidation of ω-3 fatty acids, while the peroxidation of ω-6 fatty acids produces pentane, which can also be measured in exhalate. However, ethane and pentane appear to behave differently under different conditions. Notably, isoprene may comigrate with pentane on some packed columns, making changes in pentane difficult to assess. For this reason, and because ethane is metabolized by the liver more slowly and has lower solubility coefficients in tissues than pentane, we chose ethane as a marker for lipid peroxidation in our studies.

Cigarette smokers have been shown in several studies to have reduced plasma levels of beta carotene, vitamin E, and vitamin C, and lung lining fluid levels of vitamin E, possibly because of increased utilization of these antioxidants in neutralizing smoking-induced free radicals. Supplementation may elevate these antioxidants to levels that more successfully attenuate the increased oxidative insult. The range of baseline plasma vitamin E in our study (0.62 to 1.37 ng/dL) is comparable to other published values (0.5 to 1.6 mg/dL). Our baseline beta carotene range (10 to 183 ng/mL) is also comparable to published ranges for smokers in some recent work. Plasma levels of beta carotene in the literature vary widely because of differences in diet and the proportions of smokers in each study. If “normal” levels are defined as 100 ng/mL or greater, we found that 4 of our patients had subnormal plasma values: in another recent study, 62% of smokers had subnormal baseline beta carotene levels.

When smokers were analyzed individually for changes in exhaled ethane after antioxidant supplementation, there was a significant reduction in eight of ten subjects. In the remaining two, one had a definite increase, while the other showed little change. In a well designed placebo-controlled study of 4 weeks of beta carotene supplementation by Allard and colleagues, there was a significant decrease in mean pentane, but a nonsignificant decrease in mean ethane (from 12.9 to 10.4 pmol-kg−1 min−1) in smokers. However, there are important differences between our study and theirs. First, we gave vitamin C and vitamin E along with beta carotene. Princen and colleagues found that vitamin E protected low-density lipoprotein polyunsaturated fatty acids from cigarette smoke-induced oxidation, while beta carotene did not. Therefore, vitamin E, C, or an additive effect of combined antioxidants may more effectively decrease peroxidation than beta carotene alone. Second, our absolute ethane levels are much lower than those measured by Allard and colleagues. Although the precise reason(s) for this is not clear, one possibility is that we routinely measure and subtract ethane levels in the “hydrocarbon-free” inspire (which in our experience can vary considerably among different lots) from the total levels obtained from the patient. In fact, the absolute decrease of mean ethane in our study, 1.16 pmol-kg−1 min−1, is similar to the decrease of 2.5 pmol-kg−1 min−1 observed by Allard et al. The wide range of normal ethane production in the literature may reflect similar methodologic differences.

We found no relationship between vitamin E or beta carotene levels and exhaled ethane. Since ethane production may reflect an imbalance between an oxidative load (ie, cigarette smoke) and multiple endogenous antioxidants, it is unlikely that a relationship exists for a single vitamin antioxidant. Furthermore, there may be maximum thresholds of antioxidant potentials above which further supplementation will have no benefit. Our supplemented levels very likely exceed these threshold. Finally, it is likely that organ-localized antioxidants play a greater role in blocking free radical-mediated injury than do antioxidants in plasma. Pacht and colleagues found undetectable levels of vitamin E in the alveolar lining fluid of five of seven smokers.
compared with nonsmokers, despite similar serum levels of the vitamin.

The correlation between ethane production and pack-years deserves comment. Even if exhaled ethane reflects a balance between the degree of inflammatory insult (eg, cigarette smoking) and the levels of endogenous antioxidants, we did not expect to be able to demonstrate a relationship in our small study. Because ethane represents an acute “point in time” indicator of ongoing inflammation, it is possible that pack-years serves as a surrogate marker for current tobacco use. In fact, we did find a correlation between pack-years and packs per day: a much larger study will be necessary to allow adjustment for confounding by these related variables. We hypothesize that the correlation between pack-years smoked and exhaled ethane was present only for postsupplementation ethane and tobacco use because supplementation may have “levelled the playing field.” Specifically, by providing exogenous vitamins, we may have minimized the effect of interindividual variability in antioxidant potential, allowing a dose-dependent effect of the insult (tobacco use) to become apparent.

Finally, we also noted a very strong inverse relationship between the change in ethane over the 3-week trial and the FEV1. It is conceivable that individuals with a preserved FEV1 more easily assimilate higher lung levels of micronutrient antioxidants, which in turn leads to decreased ethane production. To our knowledge, this issue has never been examined. Conversely, the ability to rapidly correct smoking-induced lung lipid peroxidation (ie, to decrease ethane production) may explain the preserved FEV1. A larger trial examining this relationship should include other measures of free radical activity, eg, conjugated diene levels in lung tissue or lavage, and more objective measures of tobacco consumption.

In conclusion, our study has shown that elevated exhaled ethane in smokers can be reduced by 3 weeks of micronutrient antioxidant supplementation. Although not a substitute for smoking cessation, these agents may attenuate smoking-related lung injury in some individuals. Additional studies are warranted to further investigate these relationships.

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