Adaptation of Lung Antioxidants to Cigarette Smoking in Humans*

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We investigated the effect of free radical scavengers, micronutrient antioxidants, on antioxidant enzyme activities in cigarette smokers. We measured the intracellular superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities and vitamin E and β-carotene levels in the bronchoalveolar cells of 14 smokers before and after 6 weeks of supplementation with vitamins E and C and β-carotene. Eight nonsmokers served as control subjects. CAT and GPx activities were higher in BAL cells from smokers compared with nonsmokers (20.5±2.3 vs 9.6±1.3 U/10⁶ cells; p=0.027; 0.90±0.10 vs 0.46±0.12 U/10⁶ cells; p=0.049, respectively), while there was no difference in SOD activity between the two groups. Likewise, vitamin E and β-carotene concentrations were noticeably higher in smokers’ lung lavage cells (403.3±11.0 in smokers vs 16.6±1.3 ng/10⁶ cells in nonsmokers, and 1.23±0.21 in smokers vs 0.15±0.04 ng/10⁶ cells in nonsmokers, respectively). The serum levels of vitamin E and C and β-carotene increased by 2.0-, 1.6-, and 8.9-fold in smokers after supplementation, which were similar to nonsmokers. Similarly, BAL cell vitamin E increased from 403.3±11.0 to 477.4±97.7 ng/10⁶ cells and β-carotene increased from 1.23±0.21 to 4.32±0.45 ng/10⁶ cells (p<0.05). Despite increased concentrations of vitamins in serum as well as β-carotene levels in BAL cells, there was no significant down regulation in SOD, CAT, or GPx activities in the lung lavage cells. These data suggest that augmentation of micronutrient antioxidants in smokers and nonsmokers does not appear to have an effect on antioxidant enzyme activities, suggesting a differential regulation of these defenses.

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Key words: β-carotene; catalase; glutathione peroxidase; lung antioxidants; superoxide dismutase; vitamin C; vitamin E

Abbreviations: CAT=catalase; GPx=glutathione peroxidase; NADH=the reduced form of nicotinamide adenine dinucleotide; NADPH=nicotinamide adenine dinucleotide phosphate; SOD=superoxide dismutase

Oxidant-mediated lung injury is important in the pathogenesis of cigarette smoke-induced lung disease. BAL in smokers is characterized by increased numbers of alveolar macrophages and neutrophils that exhibit enhanced oxidative metabolism and release of superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂). Oxidants present in cigarette smoke or released by inflammatory cells are capable of directly damaging cell components. These molecular alterations result in tissue injury in the form of bronchitis, bronchiolitis, and emphysema. In response to cigarette smoking, alveolar macrophages exhibit increases in total superoxide dismutase (SOD) and catalase (CAT) activities and glutathione levels when compared with nonsmokers. Similarly, erythrocytes from smokers contained elevated CAT activity and glutathione levels compared with nonsmokers. Likewise, alveolar macrophages from hamsters exposed to cigarette smoke demonstrated increases in CAT and SOD (primarily Cu-Zn SOD) activities without a change in glutathione peroxidase (GPx) activity when compared with control animals.

The purpose of the present study was to determine whether adaptive responses in enzymatic antioxidants can be modulated by augmentation of micronutrient free radical scavengers such as vitamin E and C and β-carotene. Our findings indicated that the augmentation of micronutrient antioxidants did not result in a decrease in antioxidant enzymes activities despite increased serum and cellular concentrations of the vitamins.

Materials and Methods

Subjects

The study population consisted of 14 asymptomatic smokers (age, 36±6 years) and 8 nonsmokers (age, 26±5 years). “Smoker” was defined by a history of cigarette smoking of at least 7.5 pack-years and current consumption of at least 1 pack per day. “Nonsmoker” was defined by a lifetime history of less than 1 pack-year more than 15 years prior to the study. Exclusion criteria included: (1) history of any active or inactive pulmonary or nonpulmonary disease; (2) previous or concurrent use of prescription medications, hormones, or vitamins; and (3) upper respiratory tract infection within the past 4 weeks prior to the study. Subjects were screened by a physician-administered questionnaire and all subjects signed an informed consent form.
consent approved by the Yale University Human Investigation Committee.

Study Design

The enzymatic activities of SOD, CAT, and GPx were measured in BAL cells of smokers and nonsmokers at baseline (after 1 week of placebo) and after 6 weeks of supplementation with micronutrient antioxidants. The study was conducted as a single-blind, placebo-controlled trial of the effect of placebo vs antioxidant vitamin supplementation. The antioxidant vitamins consisted of vitamin C (500 mg ascorbate), vitamin E (400 IU α-tocopherol), and β-carotene (7.5 mg) as a single capsule to be taken 3 times per day and indistinguishable from placebo (both from Hoffman-La Roche, Nutley, NJ).

Baseline serum samples were obtained before and after 1 week of placebo and baseline BAL, and was obtained after 1 week of placebo in both groups. After 6 weeks of dietary supplementation with vitamin C (1,500 mg/d), vitamin E (1,200 IU/d), and β-carotene (22.5 mg/d), posttreatment serum and BAL samples were obtained. Usual patterns of smoking were maintained throughout the study period. Compliance was assessed by weekly follow-up, collection of unused study drug, and measurement of serum vitamin levels.

BAL

Fiberoptic bronchoscopy and BAL were performed using standard methods as previously described. After topical anesthesia with 2 to 4% lidocaine, a flexible fiberoptic bronchoscope (Pentax Corp; Orangeburg, NY) was passed orally until a wedge position was achieved in a right middle lobe subsegment. Lavage was performed with 200 mL of 0.9% NaCl in 50-mL aliquots and returned fluid was kept on ice until further processing as previously described. Briefly, soluble and cellular components were separated by centrifugation (500 g for 15 min at 4°C); cells were washed once with Hank’s balanced salt solution without Ca2+ or Mg2+ (GIBCO; Grand Island, NY) and counted with a hemacytometer. Viability was assessed by 0.4% trypan blue exclusion. Cell differential count was performed by counting 500 cells in a Giemsa-type (Diff-Quick)-stained (American Hospital Supply; McGraw Park, Ill) aliquot of cell suspension. Cells were subsequently stored in aliquots under N2 at −70°C until further analysis.

Intracellular Antioxidant Enzyme Activities

Aliquots of cells were thawed, exposed to 0.25% sodium cholate for 5 min, lysed by sonication (5×10-s bursts), and centrifuged at 20,000 g for 5 min (Brinkman centrifuge 3200) to remove cellular debris. Enzyme assays were organized to minimize systematic error due to interassay variability masking or enhancing any real effect of smoking status or dietary antioxidant treatment. For each enzyme assay, standard curves were run using the same lot of enzyme standard under the same assay conditions (22°C, stated pH) and sample enzyme activities were read from their respective standard curves to adjust for any remaining interassay variability. Samples were run in triplicate. All solutions were made up in double-distilled water. SOD, CAT, GPx, diaphorase, the reduced form of nicotinamide adenine dinucleotide (NADH), H2O2, nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase, and glutathione-containing reagents were made up fresh on the day of each assay.

SOD Activity

SOD was assayed using microbial NADH diaphorase (Boehringer-Mannheim; Indianapolis) as the source for O2 which is detected spectrophotometrically by the oxidation of hydroxyamine-containing nitrite. Aliquots of SOD standard (bovine SOD; Sigma Chemical Co; St. Louis) or sample were incubated for 15 min in the presence of 100 mmol/L air-saturated phosphate buffer (pH 7.8), 0.5 mmol/L hydroxylamine, 0.2 mmol/L anthraquinone, 750 U/L diaphorase, 0.5 mmol/L NADH (sodium salt; Sigma Co), and with or without 1.5 mmol/L KCN (all final concentrations). Subsequently, aliquots of the incubation mixture were monitored for a color reaction at 540 nm after 20 min of incubation with equal volumes of 200 mmol/L air-saturated phosphate buffer, 1% (w/v) sulfanilamide in 25% (v/v) HCL, and 0.02% (w/v) naphthylethylendiamine-dihydrochloride. By convention, 1 enzyme unit of SOD was defined by a 50% inhibition of the detector reaction.

CAT Activity

CAT activity was measured by following the decomposition of H2O2 at 240 nm. The reaction mixture consisted of 19 mmol/L H2O2 as calculated by a molar extinction coefficient of 43.6 M−1 cm−1 in 50 mmol/L potassium phosphate buffer (pH 7.0), in the presence of known concentrations of CAT standard (bovine liver, 2 x crystallized, Sigma Co) or sample. Units of CAT activity in the samples were read directly from the standard curve.

GPx Activity

GPx activity was measured according to Paglia and Valentine. The rate of glutathione oxidation by H2O2 was monitored using 0.15 mmol/L H2O2, 1.0 mmol/L glutathione (Sigma Co), 0.15 mmol/L β-NADPH (tetrasodium salt; Sigma Co), 0.24 U/mL glutathione reductase (yeast; Sigma Co), 100 mmol/L potassium phosphate buffer containing 1.0 mmol/L EDTA, and 1.0 mmol/L sodium azide in the assay mixture at a pH of 7.0. The decrease in absorbance at 340 nm as NADPH is converted to NADP is proportional to the GPx concentration, using a molar extinction coefficient of 6.22×106 M−1 cm−1 for NADPH. Known concentrations of GPx (bovine erythrocytes; Calbiochem; La Jolla, Calif) were used to generate a standard curve. Background absorbance was subtracted. Units of GPx activity in the samples were read directly from the standard curve.

Cell Lysate Protein

Protein in cell lysate was measured according to Bradford using bovine serum albumin (Sigma Co) as the standard.

Assays of Antioxidant Vitamins

Vitamin C was measured spectrophotometrically in serum using 2,6 dichlorophenol-indophenol as the indicator, as previously described. Vitamin E and β-carotene, in serum and BAL cell lysates, were measured by high-pressure liquid chromatography according to Craft et al. The high-pressure liquid chromatography system consisted of two separate isocratic systems operating simultaneously. The columns were either a Supelcosil Cis 5 μm (Supelco, Bellefonte, Pa) or a 250×4.6 mm Altex Ultrasphere Cis 5 μm, paired with an NH2 guard column (Alltech; Deerfield, Ill). The mobile phase for vitamin E analysis consisted of 100% methanol; that for β-carotene analysis was acetonitrile/methylene chloride/methanol (70%/20%/10%, respectively, by volume). Standard curves were established using known concentrations of α-tocopherol and β-carotene.

Statistical Methods

Student’s t test for unpaired samples or Mann-Whitney test were used to analyze the effect of smoking (ie, nonsmoker vs smoker). Paired t test or Wilcoxon signed rank test were used where appropriate to analyze the effect of dietary antioxidant supplementation in smokers and nonsmokers (ie, baseline vs treated). Differences were considered significant if p<0.05.
Table 1—Subject Characteristics, Serum Vitamins, and BAL Composition at Baseline

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>26±5</td>
<td>36±8*</td>
</tr>
<tr>
<td>Smoking, pack-year</td>
<td>0.1±0.1</td>
<td>24.0±4.3*</td>
</tr>
<tr>
<td>Serum vitamins</td>
<td></td>
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</tr>
<tr>
<td>Vitamin C, mg/dL</td>
<td>0.78±0.21</td>
<td>0.94±0.18</td>
</tr>
<tr>
<td>Vitamin E, mg/dL</td>
<td>0.67±0.04</td>
<td>1.06±0.07*</td>
</tr>
<tr>
<td>β-carotene, pg/dL</td>
<td>11.8±3.3</td>
<td>7.9±1.2</td>
</tr>
<tr>
<td>BAL characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lavage recovery, %</td>
<td>73±4</td>
<td>55±3*</td>
</tr>
<tr>
<td>Total cells, x10^6</td>
<td>14.0±3.3</td>
<td>55.1±12.2</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>90.9±2.4</td>
<td>95.9±0.6*</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>8.1±2.3</td>
<td>3.2±0.5*</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>0.7±0.2</td>
<td>0.7±0.1</td>
</tr>
</tbody>
</table>

*p<0.05.

**RESULTS**

**BAL Composition**

Table 1 shows the characteristics of the subjects and the cellular composition of BAL at baseline. The total cell count was markedly increased in smokers compared with nonsmokers. The total and percentage of alveolar macrophages in smokers were significantly higher than in nonsmokers. This increase in macrophage numbers was at the expense of decreased lymphocytes. The total number of neutrophils was increased in smokers. Dietary supplementation with vitamins C and E and β-carotene did not alter BAL cellular characteristics in either nonsmokers or smokers (data not shown).

**Serum Antioxidant Vitamins**

There was no significant difference in serum vitamin C or β-carotene levels between smokers and nonsmokers at baseline after placebo (Table 1). However, smokers had higher serum levels of vitamin E than nonsmokers. Serum levels of vitamins C and E and β-carotene increased significantly in both groups after supplementation (Table 2). The serum levels in nonsmokers for vitamins E, C, and β-carotene increased by 2.4-, 1.6-, and 6.6-fold, respectively. In smokers, the corresponding serum levels increased by 2.0-, 1.6-, and 8.9-fold. The increments in serum vitamin levels after supplementation were comparable in smokers and nonsmokers.

**BAL Cell Antioxidant Vitamins**

As shown in Table 2, the cellular content of vitamin E was more than 20-fold higher in smokers at baseline (403.3±51.0 ng/10^6 cells) compared with nonsmokers (16.6±3.5 ng/10^6 cells; p=0.001). Similarly, the cellular content of β-carotene in smokers was 8-fold higher than that of nonsmokers (1.23±0.21 ng/10^6 cells vs 0.15±0.04 ng/10^6 cells; p=0.0005). After dietary supplementation, cellular content of β-carotene increased in both smokers and nonsmokers with a modest (18%) but statistically insignificant change in vitamin E content (Table 2). Vitamin C was not measured in cell lysates because of insufficient samples.

**BAL Cell Antioxidant Enzymes**

Effect of Smoking: Smoking was associated with significantly increased activities of CAT and GPx in BAL cells (Table 2). Cells from smokers had more than twice the level of CAT activity (20.5±2.3 U/10^6 cells vs 9.6±1.3 U/10^6 cells; p=0.027) and nearly twice the level of GPx activity (0.90±0.10 U/10^6 cells vs 0.46±0.12 U/10^6 cells; p=0.049) compared with those from nonsmokers. These differences were observed whether they were expressed per number of cells or protein content (data not shown). There was no significant difference in SOD activity between smokers and nonsmokers.

Effect of Dietary Antioxidant Supplementation: The vitamin supplementation had no significant effect on the SOD or GPx enzyme activities in BAL cells of smokers. However, CAT activity increased by 55% in smokers’ cells after supplementation (Table 2). In nonsmokers, SOD activity increased by 53% (p=0.04), with no significant change in CAT or GPx activities. In smokers and at baseline, there was an inverse relationship between serum vitamin E concentration and BAL cells’ CAT activity; r=0.62; p<0.05 (Fig 1). This inverse relationship was also noted between BAL cells’ vitamin E level and CAT activity with marginal statistical significance. No similar correlation was found between serum vitamins’ and BAL cells’ antioxidant enzymes in nonsmokers. Moreover, there was no cor-

![Figure 1. Relationship between serum vitamin E concentrations and CAT activity of BAL cells in smokers before supplementation with vitamins.](image-url)
Table 2—Serum Vitamins, BAL Cell Vitamins, and Antioxidant Enzymes Before and After Dietary Supplementation With Vitamin C, Vitamin E, and β-Carotene

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers*</th>
<th>Smokers†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Treated</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C, mg/dL</td>
<td>0.78±0.21</td>
<td>1.22±0.24</td>
</tr>
<tr>
<td>Vitamin E, mg/dL</td>
<td>0.67±0.04</td>
<td>1.60±0.10</td>
</tr>
<tr>
<td>β-carotene, mg/dL</td>
<td>11.8±3.3</td>
<td>78.4±15.0</td>
</tr>
<tr>
<td>BAL cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E, ng/10⁶ cells</td>
<td>16.6±5.3⁺</td>
<td>39.2±13.1¹</td>
</tr>
<tr>
<td>β-carotene, ng/10⁶ cells</td>
<td>0.15±0.04⁺</td>
<td>1.08±0.53⁴</td>
</tr>
<tr>
<td>SOD, U/10⁶ cells</td>
<td>4.9±1.2</td>
<td>7.5±0.6</td>
</tr>
<tr>
<td>CAT, U/10⁶ cells</td>
<td>9.6±1.3</td>
<td>16.8±2.5</td>
</tr>
<tr>
<td>GPx, U/10⁶ cells</td>
<td>0.46±0.12</td>
<td>0.60±0.06</td>
</tr>
</tbody>
</table>

* *Nonsmokers, n=8 unless otherwise indicated.
† Smokers, n=14 unless otherwise indicated.
⁺ p<0.05.
†† p<0.05, nonsmoker vs smoker after placebo.
* p<0.05, placebo vs treated in each group.
** p<0.05, nonsmoker vs smoker after treatment.

The relation between serum and BAL cell vitamins and antioxidant enzymes after supplementation.

**DISCUSSION**

In the present study, we demonstrated the following: (1) BAL cells from smokers had higher levels of vitamin E and β-carotene compared with nonsmokers; (2) serum vitamin E level correlated inversely with BAL CAT activity in smokers; (3) BAL cells from smokers had increased CAT and GPx activities compared with nonsmokers; (4) BAL cellular concentration of β-carotene and serum levels of micronutrients increased significantly after supplementation in both smokers and nonsmokers; and (5) despite augmentation of micronutrient antioxidants in serum and BAL cells, there was no decrement in activities of antioxidant enzymes in BAL cells.

Cigarette smoking is likely to affect micronutrient status. In large population studies, plasma levels of vitamin C and β-carotene have been shown to be decreased in smokers. Nutritional deficiencies may alter the expression of antioxidant enzymes. Lungs from vitamin E-deficient rats show increased glucose-6-phosphate dehydrogenase and glutathione peroxidase activities compared with control animals, possibly to compensate for loss of antioxidant capacity. Likewise, and probably due to free radicals and oxidant burden, the alveolar macrophages of smokers had higher SOD and CAT activities than those of nonsmokers. In our study, smokers had higher GPx activities than nonsmokers. In a study by Cantin and colleagues, smokers had higher concentrations of reduced glutathione in the BAL. Another important finding in the present study was that smokers had higher levels of vitamin E and β-carotene in their BAL cells. Serum vitamin E level was higher in smokers, as noted by others. These adaptive responses to oxidant exposure appear to be regulated at different levels and involving different systems. It is known that smokers' lung lavage cells concentrate vitamin E similar to mobilization of vitamin E to the lung after ozone exposure in rats. The mechanisms by which lung cells concentrate these micronutrients are not well defined. However, in the case of vitamin E, two factors, vitamin E deficiency and oxidant exposure, appear to play an important role in increasing the cellular uptake of the vitamin. Likewise, vitamin C also tends to concentrate in the alveolar macrophages of smokers.

The mechanism(s) of the adaptive response to oxidant exposure is not well established. The evidence in the literature supports regulation of antioxidant enzymes at both transcriptional and translational levels which may be independent of the antioxidant status of the cell. In agreement with our data, Duthie and coworkers found no significant correlation among SOD, CAT, and GPx and vitamin E and C in erythrocytes of smokers. Moreover, vitamin E supplementation had no effects on erythrocyte antioxidant enzymes in this study. There are potentially several reasons as to why antioxidant supplementation was not effective in altering antioxidant enzymes: (1) the mechanism of antioxidant adaptation to oxidant burden is different for micronutrient and enzymatic antioxidants; however, this ineffectiveness of micronutrient antioxidants in altering the expression of antioxidant enzymes does not rule out involvement of the corresponding reactive oxygen intermediates and oxidants inasmuch as local concentrations may be insufficient to alter oxidant ki-
netics measurably; (2) the micronutrient antioxidants may not be efficient enough or strategically placed to totally quench the oxidants; and (3) the smokers in this study showed no significant deficiencies in serum vitamins, in part, perhaps to less intense smoking habit which may have ameliorated the oxidant burden.

In summary, we have shown that cigarette smoking in humans is associated with selective augmentation of antioxidant enzyme activities and micronutrient antioxidants in lung lavage cells as an adaptive response to oxidant stress. Enhancement of micronutrient antioxidants has no direct effect on the regulation of antioxidant enzymes. The value of these adaptive responses in protecting smokers from lung damage can be determined only by a long-term prospective study with micronutrient supplementation.

REFERENCES