The Effects of Dietary Antioxidants on NO₂- Induced Injury to Type 1 Alveolar Cells*

Michael J. Evans, Ph.D.; Linda J. Cabral-Anderson, B.S.;
Nuti F. Dekker, B.A.; and Gustave Freeman, M.D.

The mechanism by which NO₂ damages type 1 cells is thought to be oxidation of unsaturated fatty acids of the cell membranes.1-3 Protection from such oxidation has been associated with the antioxidant properties of selenium and vitamin E.4,5 Selenium is a component of the enzyme glutathione peroxidase (GP), which is thought to participate in breaking the autocatalytic chain reaction of lipid peroxidation by decomposing lipid hydroperoxides.6 Vitamin E is thought to act as an antioxidant by terminating peroxidation initiated by free radical mechanisms.7 However, Mead et al7 suggested recently that vitamin E could also protect unsaturated fatty acids during exposure to NO₂ because of its greater reactivity with NO₂ than with unsaturated fatty acids. Together, the two compounds have been shown to be more beneficial in preventing oxidation of unsaturated fatty acids than is either compound acting alone.6

The time course of vitamin E protection was demonstrated by in vitro studies concerning the oxidation of thin films of polyunsaturated fatty acid by NO₂.5,7 These studies indicate that vitamin E delays the onset of oxidation. The delay persists until the vitamin E present has been oxidized; then oxidation of the polyunsaturated fatty acid proceeds at the same rate determined in studies in which no vitamin E was added. The time course of possible protection provided by GP has not been demonstrated.

A number of studies have shown that these dietary antioxidants protect the lung from oxidant injury.5,8-10

*From the Medical Sciences Department, SRI International, Menlo Park, California.
Reprint requests: Dr. Evans, SRI International, Menlo Park, California 94025

However, the protective effect of dietary antioxidants has not been demonstrated at the cellular level. Since the type 1 cell is the alveolar cell type injured during exposure to NO₂, it should also be the cell protected by dietary antioxidants.1 According to the observations mentioned earlier, we hypothesized that dietary antioxidants may protect type 1 cells from NO₂-induced injury, but that the protection would be limited to the duration of exposure. We tested this hypothesis in rats fed diets containing different amounts of vitamin E and selenium by quantitating the amount of injury that occurred to the type 1 alveolar epithelium following sublethal exposure to NO₂ for 6, 12, 24, or 48 hours.

MATERIALS AND METHODS

One-month-old male rats (Hilltop Animals) were divided into three groups, and each group was fed one of the following diets for 10 weeks:8 group A was fed supplemental amounts of vitamin E (dl-α-tocopherol; 0.5 g/kg of basal diet† deficient in vitamin E) and selenium (sodium selenite; 0.1 mg/kg); group B was fed adequate amounts of vitamin E (0.05 g/kg) and selenium (0.04 mg/kg). Neither vitamin E nor selenium was added to the basal diet fed to group C. Trace amounts of selenium were present in the basal diet, so group C was not considered deficient in it.

In four separate experiments, rats from the three diet groups were exposed simultaneously to approximately 15 ppm NO₂ for 6 to 48 hours and then returned to a normal atmosphere (Table 1). Details of the exposure facilities were presented previously.1 Animals were weighed before exposure began. Vitamin E levels in the tissues were determined12 for exposed and control animals in experiments 3 and 4. The selenium content of control tissues were evaluated by measuring GP levels13 in experiments 3 and 4.

Rats from each group were sacrificed from the beginning of the exposure at daily intervals through four days in experiment 1 and through five days in experiments 2-4. At 9:00 AM they were injected intraperitoneally with 3H-TdR

Table 1—Experimental Conditions

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Duration of Exposure (hr)</th>
<th>NO₂ Concentration (ppm)</th>
<th>Diet</th>
<th>Animal Weight (g)</th>
<th>Vitamin E Levels (% of control)</th>
<th>Glutathione Peroxidase Activity (% of control)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>15.4 ± 2.2</td>
<td>A</td>
<td>460 ± 20</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>426 ± 40</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>368 ± 32</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>14.8 ± 1.6</td>
<td>A</td>
<td>526 ± 46</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>504 ± 53</td>
<td>–</td>
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<td></td>
<td></td>
<td>C</td>
<td>396 ± 49</td>
<td>–</td>
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<tr>
<td>3</td>
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<td>458 ± 37</td>
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<td>C</td>
<td>331 ± 25</td>
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<td>4</td>
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<td>511 ± 36</td>
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<td></td>
<td>C</td>
<td>381 ± 34</td>
<td>-95</td>
<td>-29.4</td>
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</tbody>
</table>
have been killed with an overdose of sodium pentobarbital. Five rats were killed at each 24-hour interval in experiment 1, and three were killed at each interval in experiments 2-4. Unexposed rats served as controls. The lungs were fixed and prepared for autoradiography by previously described methods.\(^\text{14}\)

Type 1 cell injury was quantitated indirectly by determining the proliferative response of type 2 cells. The type 2 cell proliferative response is determined by the sum of type 2 cell labeling indexes (LIs) at all points from the beginning of the experiment until the LIs have returned to control levels.\(^\text{16-18}\) LIs were determined for type 2 cells from counts of at least 2,000 alveolar cells per animal.\(^\text{15}\)

**RESULTS**

**General Observations**

Body weights (mean ± SD) for rats in each of the experiments are presented in Table 1. In each experiment, rats fed the deficient diet (group C) had the lowest mean body weight. Otherwise, they appeared similar to rats fed supplemented diets (groups A and B).

**Type 2 Cell Proliferation**

**Experiment 1, 6 hours’ exposure to 15.4 ± 2.2 ppm NO\(_2\):** Type 2 cell LIs are presented in Figure 1. In each diet group the highest LIs were obtained one day after the beginning of the experiment. By the fourth day, the LIs had returned to control levels. Comparing the proliferative response among diet groups revealed no significant differences between groups B (19.7 ± 6.4) and A (23.3 ± 8.0), but group C (41.4 ± 7.1) had a significantly greater response (110%) than either group A or B (P < 0.05), indicating more extensive type 1 cell damage. Levels of vitamin E and GP activity were not determined.

**Experiment 2, 12 hours’ exposure to 14.8 ± 1.6 ppm NO\(_2\):** Again, in each diet group the highest LIs were obtained one day after the beginning of the experiment (Fig 2) and by the fourth day the LIs had returned to control levels. When the proliferative responses were compared among diet groups, there was no significant difference between group B (39.5 ± 4.7) and group A (34.0 ± 11.3), but group C (58.0 ± 8.0) had a significantly greater response (48%) than either group A or B (P < 0.05). These data indicate more type 1 cell injury in group C than in groups A or B, but the difference was not as great as that seen after 6 hours’ exposure. Levels of vitamin E and GP activity were not determined.

**Experiment 3, 24 hours’ exposure to 15.4 ± 1.9 ppm NO\(_2\):** In each diet group the LIs were highest one and two days after the beginning of the experiment (Fig 3). By the fourth day the LIs had returned to control levels. Comparing the proliferative response among diet groups revealed no significant differences among groups A (67.2 ± 6.8), B (53.0 ± 12.1), and C (59.2 ± 14.0). These data indicate that all three groups had similar amounts of type 1 cell injury. Analysis of vitamin E levels in pulmonary tissue revealed that those
in group A were 149% higher than those in group B, and those in group C were 86% lower than the levels in group B (Table 1). No significant difference in GP activity was observed between groups A and B, but GP activity was 39.5% lower in group C than in group B (Table 1).

Experiment 4, 48 hours' exposure to 15.8 ± 1.6 ppm NO₂: As in experiment 3, in each diet group the LIs were highest one and two days after the beginning of the experiment (Fig 4). By the fifth day the LIs had returned to control levels. Comparing the proliferative response of the type 2 cells among diet groups revealed no significant differences among groups A (99.7 ± 17.3), B (108.0 ± 13.3), and C (111.2 ± 45.3). Again, the data reflect no differences in the amount of type 1 cell damage among the groups. Analysis of the tissue vitamin E revealed that the level in group A was 35% greater than that in group B and that the level in group C was 95% lower than that in group B (Table 1). No significant difference was observed in GP activity between groups A and B, but GP activity in group C was 29.4% lower than that in group B (Table 1).

**Discussion**

The results of this study indicate the proliferative response of type 2 cells following injury to type 1 epithelium caused by 6, 12, 24, and 48 hours of exposure to approximately 15 ppm NO₂. Proliferation of type 2 cells reflects replacement of damaged type 1 cells, and has been shown to be a quantitative index of the amount of type 1 epithelium that has been severely damaged by NO₂. Considering the proliferative response of type 2 cells as an index of type 1 epithelial damage, we found that after 6 hours' exposure to NO₂, there was about 110% more type 1 cell damage in the lungs of rats fed a diet deficient in vitamin E (group C) than in those fed an antioxidant-adequate diet (group B). However, between the rats in group B and those given diets supplemented with vitamin E (group A), there was no difference in the amount of type 1 cell damage. After 12 hours of exposure, rats in group C also had more type 1 cell damage (about 48%) than did those in group B, and, again, the difference between groups A and B was insignificant. After 24 and 48 hours of exposure to NO₂, no significant differences were observed in the amount of type 1 cell damage among any of the three diet groups.

We interpret these data to indicate that the antioxidants in the adequate and supplemented diets (groups A and B) delayed the onset of damage to type 1 cells caused by NO₂. Comparing the indexes of type 1 cell damage in groups C and B after 6 and 12 hours of exposure, we found that group C (antioxidant-deficient diet) had an index level of 41.4 after 6 hours of exposure and group B a similar level (39.5) after 12 hours of exposure. This suggests that the protection afforded to type 1 epithelium in rats from group B by the antioxidants used in this study leads to a delay of about 6 hours in the onset of damage. However, the data from the 24- and 48-hour exposures showed no differences in type 1 cell injury, indicating that, following the delay, the susceptibility to injury of the rats in the groups fed antioxidant-adequate and supplemented diets rapidly came to that of the antioxidant-deprived groups.
ACKNOWLEDGMENTS: This research was supported by U.S. Public Health Service Grants HL16330, ES00842, and CA19354 from the Department of Health, Education, and Welfare. We thank Dr. D. Thomas and Dr. D. Negi for performing the vitamin E analyses and Dr. A. Brandt for the analysis of glutathione peroxidase levels in the lung.

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Alveolar Macrophage Stimulation and Population Changes in Silica-Exposed Rats*

Gerald S. Davis, M.D.; David R. Hemeneuy, Ph.D.; John N. Evans, Ph.D.; Don J. Lapanas, M.D.; and Arnold R. Brody, Ph.D.†

Silicosis is a disease of chronic granulomatous inflammation and fibrosis. The pulmonary macrophage is believed to be an important participant in both the inflammatory and fibrogenic responses in silicosis. Other workers have reported accumulations of dust-laden macrophages in laboratory animals after several months of continuous, heavy exposure to silica dust.1-4 The timing and kinetics of this macrophage response have not been defined, and the nature of this cell population has not yet been fully described. Evidence is contradictory as to whether these accumulated macrophages are injured participants in dust clearance from the lung, or are inflammatory cells responding to chronic stimulation.

Previous studies have examined limited features of the alveolar macrophage population immediately following brief dust exposure5 or after many months of continuous dust exposure.1-4 The experimental design of the present study called for limited discreet dusting, and then long-term follow-up after dusting ceased. This design may permit distinction between the potential acute toxic and the chronic stimulatory effects of silica dust.

METHODS

Respiratory disease-free Fischer 344 rats were exposed in horizontal flow dusting chambers to respirable alpha-quartz at 100 mg/m³ for six hours per day on eight consecutive days. Groups of animals were sacrificed for study 60, 90, 150, 180, and 320 days after the initial eight-day silica aerosol exposure. At each time point paired control rats were sacrificed for parallel studies. At times of sacrifice groups of animals underwent determination of pulmonary function, lung tissue was fixed in situ by vascular perfusion with Karnovsky’s solution, or lungs were excised for pulmonary lavage. Measurement of lung compliance and vital capacity were performed in situ in a small animal plethysmograph.6 Alveolar macrophages from silica dusted and control rats were recovered by pulmonary lavage of excised lungs using standardized volumes of phosphate buffered saline solution. Total lavage cell yield, differential cell count, and cell viability were determined on each specimen. Lavage macrophage oxygen consumption was measured by Clark electrode during phagocytosis of zymosan yeast particles. These preparations were also scored for the percentage of

*From the Departments of Medicine, Physiology and Biophysics, Pathology, and Civil Engineering, College of Medicine, University of Vermont, Burlington, VT. This work was supported by Grant HL-14212-SCOR from the National Heart, Lung, and Blood Institute.

†Presently at Laboratory of Pulmonary Function and Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.

Reprint requests: Dr. Davis, Pulmonary Unit, G100 C-317, College of Medicine, UVM, Burlington, Vermont 05405