Nutrient Interactions and Toxicity

Vitamin A Depletion Induced by Cigarette Smoke Is Associated with the Development of Emphysema in Rats1,2

Ting Li, Agostino Molteni,* Predrag Latkovich,* William Castellani* and Richard C. Baybutt3

Department of Human Nutrition, Kansas State University, Manhattan, KS 66506 and *Department of Pathology and Pharmacology, Medical School of the University of Missouri at Kansas City, Kansas City, MO 64108

ABSTRACT We showed previously that vitamin A deficiency per se causes emphysema. Benzo(a)pyrene, a constituent in cigarette smoke, induces vitamin A depletion when administered to rats; therefore, we tested the hypothesis that cigarette smoke induces vitamin A depletion, which is associated with the development of emphysema. Male weanling rats were fed a purified AIN-93G diet and divided into two groups. The experimental group was exposed to cigarette smoke from 20 nonfiltered commercial cigarettes/d for 5 d/wk, whereas the control group was exposed to air. After 6 wk, tissues were collected for histological and biochemical analyses. Retinol levels were measured in serum, lung and liver. The trachea, lung and liver were examined for histological changes. Vitamin A levels decreased significantly in serum, lung and liver of smoke-treated rats. Histological examination revealed the presence of interstitial pneumonitis along with severe emphysema. There was a significant inverse relationship between vitamin A concentration in the lung and the severity of emphysema (r = −0.69 and P < 0.03). Detachment or hyperplasia (and metaplasia) of the tracheal epithelium and liver vacuole formation also were evident in the smoke-treated rats. The results of this research indicate that exposure to cigarette smoke induces vitamin A depletion in rats, which is associated with the development of emphysema. J. Nutr. 133: 2629–2634, 2003.

KEY WORDS: • smoking • vitamin A deficiency • lung • trachea • inflammation

Cigarette smoke is the major cause of chronic obstructive pulmonary disease, a leading cause of death in the United States (1). In these patients, two respiratory conditions are most closely associated with cigarette smoking, chronic bronchitis and emphysema.

Emphysema is a destructive disease of the pulmonary parenchyma characterized by large air spaces in the lungs, which are associated with a smaller number of alveoli. In 1964 the U.S. Surgeon General first reported a potential relationship between smoking and emphysema, and the connection was strengthened by numerous epidemiologic and animal studies (2). Having established that cigarette smoking is the leading cause of emphysema, the focus has shifted to determining how smoke-induced emphysema develops. Among the proposed mechanisms, the protease/antiprotease and oxidant/antioxidant hypotheses are popular. The major tenant of the protease/antiprotease theory is that cigarette smoking results in an imbalance between the antiprotease, α-1 antitrypsin and elastase, resulting in increased activity of elastase. The active elastase catabolizes the matrix protein elastin, which results in a reduction in the number of alveoli and the creation of larger air spaces or areas of emphysema.

The basis for the oxidant/antioxidant hypothesis is that the oxidants in the cigarette smoke deplete the antioxidant supply in the lung and cause oxidative injury to the tissues, leading to emphysema. During exposure to cigarette smoke, large amounts of oxygen free radicals are generated; these radicals could damage the lipid components of the cell membranes as well as the matrix components of the lung (3). Destruction of lung matrix, especially elastin, may lead to emphysema. The precise chemical and biochemical mechanisms for the injuries induced by cigarette smoke have not been thoroughly explored.

An alternative hypothesis for the development of cigarette smoke-induced emphysema attributes the disease to the consequences of vitamin A deficiency. Vitamin A and related retinoids regulate normal lung development, maturation and maintenance of the alveolar epithelium (4). Benzo(a)pyrene (BP),4 a chemical compound found in cigarette smoke, administered to rats, induces vitamin A depletion in the lung and liver (5). Previously, we found that vitamin A deficiency per se

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caused emphysema in rats (6). In addition, administration of all-trans-retinoic acid (RA), an active metabolite of vitamin A, reversed experimental elastase-induced emphysema by increasing the number of pulmonary alveoli (7). Furthermore, postnatal treatment with RA increased the number of alveoli relative to those in the lungs of control rats (8). Moreover, RA was found to increase elastin in rat lung fibroblast culture (9). Taken together, these studies suggest that vitamin A plays a critical role in preventing the development of emphysema. Thus, we hypothesized that cigarette smoke may induce vitamin A depletion, which in turn contributes to the development of emphysema.

The purpose of this study was to determine whether lung vitamin A status is altered in response to cigarette smoke exposure and whether this is associated with the development of emphysema. We evaluated the histological appearance of the lung and measured the levels of vitamin A (retinol) in the lung and serum after smoke exposure in comparison with air-exposed controls. Because the liver is the major site for vitamin A storage, we also measured liver vitamin A levels and evaluated the histological appearance of liver in response to cigarette smoke exposure.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley weanling rats (Harlan Sprague Dawley, Indianapolis, IN) were housed individually in stainless steel cages at room temperature, ~24°C under a 12-h light:dark cycle (light 0600–1800 h) with a relative humidity of ~50%. Animal care and use were approved by the Institutional Animal Care and Use Committee of Kansas State University. Rats were fed a standardAIN-93G diet (10) and consumed food and water ad libitum. Food intake was recorded daily and body weight was measured once each week.

Cigarette smoke exposure conditions. Rats were randomly distributed into two groups, those exposed to cigarette smoke (SM) or those exposed to air (CT, control). The smoke-exposure group was exposed to 20 commercial nonfiltered cigarettes/d, 5 d/wk. The smoke treatment was modeled after previously published studies (11). Rats were placed in a plastic chamber that was 65 cm long, 50 cm wide, and 45 cm high with three holes, two for holding the cigarettes at one end of the chamber and another on the opposite side of the chamber connected to a tube attached to a Leseon vacuum pump (model #A6C17E201.1, Labconco, Kansas City, MO). The rats were exposed to 5 min of smoke followed by 5 min of air until all 20 cigarettes were consumed, which took ~1.5 h. CT rats were placed in another chamber but exposed to air only. The extent of exposure to rats to cigarette smoke was ascertained by measuring total particulate matter (TPM) inhaled. Briefly, TPM was determined by using Pall Gelman 25-mm filters (Pall, Filterite Division, Timonium, MD) throughout the area in which the rats were located in the smoke chamber. The weight difference of the filter paper was determined after the full treatment duration (20 cigarettes). The mean concentration of TPM was 13.1 ± 3.4 (SEM) μg/L, with a total exposure of 467 ± 120 μg/d for the SM group and 0.0 μg/L and 0.0 μg/d for the CT group (air exposure). As a measure for the gaseous phase of the smoke, the percentage of blood carboxyhemoglobin was determined to be 3.68 ± 0.08% for the SM group and 1.36 ± 0.04% for the CT group.

Serum and tissue collection. On the termination day, rats were anesthetized with sodium pentobarbital intraperitoneally. Blood was drawn from the abdominal aorta and the serum was separated and stored at −70°C until analysis. Liver, heart and lung were removed at necropsy and weighed. A lobe of liver was collected and fixed with 10% buffered formalin for 1 wk. Another lobe of the liver was immediately frozen in liquid nitrogen and stored at −70°C until analysis. The left lobe of lung was inflated with uniform pressure (~9 cm H2O) with 10% buffered formalin and placed in the same solution. The right lung was frozen in liquid nitrogen immediately and stored at −70°C. A section of the trachea was also fixed in formalin for histological analysis.

Morphological assessment. After the tissues were fixed in formalin, they were embedded with paraffin and cut in the sagittal plane. The sections were stained by hematoxylin and eosin and examined by light microscopy. Masson's trichrome staining for collagen and Weigert's staining for elastin were also applied to representative samples from each group. A semiquantitative evaluation of histological damage to the lung, liver and trachea was carried out according to previously published methods (12,13). Briefly, two pathologists, unaware of the treatments, subjectively evaluated the tissues for specific types of injury. Mean scores were assigned to each tissue. Scores ranged from "10" (presence of definite damage) to "40" (very severe damage). A score of "5" indicated some areas of the sections had the specific damage but some areas were without damage. Tissue with normal appearance received a score of "0". The means of the assigned values for each group were reported.

Retinol analysis. Serum, lung and liver were analyzed for total retinol concentration by the method of Ross (14). The samples were extracted, saponified and quantified by HPLC analysis. The details were published previously (6). The mean retinol recovery rate was 91% for liver, 93% for lung and 90% for serum.

Serum enzymes. Serum alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), aspartic aminotransferase (AST) and lactate dehydrogenase (LDH) were measured by the Dade Dimension R&L analyzer (Newark, DE) using the manufacturer's reagent.

Statistical analysis. All data are presented as means ± SEM. Treatment effects were analyzed using one-way ANOVA with the general linear model procedure (SAS Institute, Cary, NC). Linear regression analysis (SAS Institute) was used to determine the correlation between lung vitamin A concentration and the severity of emphysema. In all analyses, P < 0.05 was considered significant.

For the histological analysis, all statistical evaluations were performed with the Asyste add-in for Microsoft Excel (DDU Software, Leeds, UK). Probabilities were derived by Kruskal-Wallis one-way ANOVA as determined by software. Probability estimates were derived by the Mann-Whitney U test from the statistic determined by the software referenced to tables of critical values of the U test for small sample size (15).

RESULTS

General observations. None of the rats died before the termination day. Daily food intake did not differ between SM and CT, nor did mean weight gain/wk (CT = 34.75 ± 6.05 g/wk; SM = 31.07 ± 5.33 g/wk). Initially, there was some vasculitis and hemorrhaging in the lungs of SM rats at wk 4, but this was not observed at 6 wk (data not shown). Lung, heart and liver to body weight ratios in SM rats were significantly greater than in CT rats (Table 1).

Histopathological evaluations. The lungs of CT rats killed at wk 6 were essentially normal (Fig. 1A). Interstitial pneumonia and severe diffuse emphysema were observed histologically in the lungs of SM rats (Fig. 1B and C, respectively). These changes were quantified in relation to the severity of the lesion and summarized (Table 2). The inflammatory process was characterized by alveolar septal thickening, septal infiltration by erythrocytes and chronic inflammatory cells (mostly

| Table 1 |
| Body and relative organ weights of rats exposed to cigarette smoke (SM) or air (CT) for 6 wk1 |
| Treatment | Body weight | Lung weight | Heart weight | Liver weight |
| SM | 224 ± 7.3 | 0.60 ± 0.04 | 0.41 ± 0.01 | 3.05 ± 0.05 |
| CT | 244 ± 12 | 0.52 ± 0.01 | 0.37 ± 0.01 | 2.84 ± 0.06 |

1 Values are mean ± SEM, n = 4, CT; n = 6, SM. * Different from control (CT), P < 0.05.
macrophages), with a scattered presence of these cells within the alveolar space. A number of small caliber arteries also showed mild vasculitis, characterized by thickening of the artery wall (mostly the media), some periadventitial edema with few scattered macrophages, and a modest reduction of the arterial and arteriolar lumen. Pneumonitis was present along with diffuse areas of emphysema in the lung of SM rats, which involved the whole organ (Fig. 2). Elastin stain also was less evident in SM rats relative to controls (Table 2).

Large areas of detached epithelium and hyperplasia of the tracheal epithelium were seen in SM rats (Fig. 3B) compared with CT rats (Fig. 3A). There also were a few areas of squamous metaplasia. The mean assigned score for detached epithelium and hyperplasia were 33.7 ± 13.7 and 12.5 ± 1.7 for the SM rats, respectively, which was significantly higher than the CT rats (1.3 ± 1.3 and 1.3 ± 1.3, respectively). Similar conditions were noted in the large bronchi of the same rats. In addition, inflammatory cells and cellular debris were observed.

The liver of SM rats showed moderate vacuolization of the hepatocytes (8.3 ± 1.7) that was greater (P < 0.05) than the mean assigned score for the CT group (1.3 ± 1.3). Fibrosis was not apparent, but mild deposition of collagen was observed in the portal triad of these rats. Control rats did not have any abnormalities, with the exception of minimal fatty infiltration in one rat.

**Serum, lung and liver levels of retinol.** After 6 wk of smoke exposure, retinol levels were significantly decreased in SM rats in serum, lung and liver (Table 3). There was a significant inverse correlation (r = -0.69, P < 0.03, n = 10) between the retinol concentration in the lung and the severity of the emphysema in all rats (Fig. 4).

**Biochemical analysis of serum.** After 4 wk, serum GGT and AST were significantly higher in the SM group compared with the CT group (data not shown), but returned to control values after 6 wk. The values for ALT and LDH for the SM group did not differ from controls throughout the study.

**DISCUSSION**

In the present study, we investigated histological changes caused by exposure to cigarette smoke and measured vitamin A levels in the exposed rats. Our data demonstrated that exposure to cigarette smoke: 1) induced vitamin A depletion with diffuse areas of emphysema in the lung of SM rats, which involved the whole organ (Fig. 2). Elastin stain also was less evident in SM rats relative to controls (Table 2).

Large areas of detached epithelium and hyperplasia of the tracheal epithelium were seen in SM rats (Fig. 3B) compared with CT rats (Fig. 3A). There also were a few areas of squamous metaplasia. The mean assigned score for detached epithelium and hyperplasia were 33.7 ± 13.7 and 12.5 ± 1.7 for the SM rats, respectively, which was significantly higher than the CT rats (1.3 ± 1.3 and 1.3 ± 1.3, respectively). Similar conditions were noted in the large bronchi of the same rats. In addition, inflammatory cells and cellular debris were observed.

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**TABLE 2**

Semiquantitative evaluation of the morphological changes observed in the lung of rats exposed to cigarette smoke (SM) or air (CT) for 6 wk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pneumonia</th>
<th>Emphysema</th>
<th>Elastin deposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>2.5 ± 1.4</td>
<td>2.5 ± 1.4</td>
<td>10.0 ± 0.0</td>
</tr>
<tr>
<td>SM</td>
<td>15.0 ± 3.2*</td>
<td>16.7 ± 2.1*</td>
<td>5.8 ± 0.8*</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 4, CT; n = 6, SM. Numerical values were assigned according to the observer’s impression of the severity of the tissue injury. * Different from control (CT), P < 0.05.

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**FIGURE 1** Representative histological sections of the lung of normal and cigarette-exposed rats. (A) Tissue section of the lung of a control rat. No abnormalities were found. (B) Section of an area in the lung of a rat exposed to cigarette smoke for 6 wk. The alveolar septa were thickened significantly with infiltration of inflammatory cells. Diffuse interstitial pneumonia was present. RBC were spread in the septa and the alveolar lumen. (C) Histological section of another area of the lung of the same rat depicted in Fig. 1B. The alveolar wall was thinner than normal. Emphysematous areas, dilation of many alveolar spaces and destruction of the septa walls were evident. RBC and a few chronic inflammatory cells were also present. Staining: trichrome; magnification: X400.
in the lung, serum and liver; 2) resulted in pulmonary inflammation and emphysema with vitamin A concentration in the lung significantly inversely related to the severity of emphysema; 3) reduced elastin staining in the lung; 4) caused detachment or hyperplasia of tracheal epithelium; and 5) increased vacuole formation (fatty infiltration) in the liver.

To our knowledge, this study provides the first evidence that cigarette smoke inhalation by rats decreases lung, serum and liver retinol levels. Because food intakes did not differ between the groups, the depletion appeared to be caused by cigarette smoke per se. These findings are supported by others (5,16) who administered BP, a constituent of cigarette smoke, to rats, causing vitamin A–deficient lungs and livers. In addition, others have found that intraperitoneal administration of tobacco extract or a tobacco constituent, N′-nitrosonornicotine, decreased the hepatic pool of vitamin A (17). Furthermore, exposure to cigarette smoke induces the production of the lung and liver cytochrome P450 isoforms, CYP1A1 and CYP1A2 (18,19), which increase the catabolism of RA (18,20) and may lead to vitamin A depletion. Although any of these may have depleted vitamin A levels, the precise mechanism remains to be determined.

The vitamin A depletion in our smoke-exposed weanling rats is consistent with other smoke exposure studies. When adult ferrets were exposed to cigarette smoke, retinoid catabolism increased, resulting in significantly lower levels of RA in the lung (21,22). Despite this increased catabolism, lung retinol concentrations were not significantly altered in that study. Although we did not measure RA in the present study, the decreased tissue retinol concentrations suggest an increased retinoid catabolism. The reason why significantly lower levels of retinol were not observed in the ferret study is not known, but may be due to differences in the age of the animals. Adult ferrets have larger liver stores of vitamin A and may have compensated more effectively for losses in the lung.

The amount of smoke exposure may be another important determinant for the smoke-induced vitamin A depletion. When weanling guinea pigs were exposed to a low dose of 6 cigarettes/d for 6 wk, the levels of retinol in the lung increased (23). In contrast, when we exposed weanling rats to a higher dose of 20 cigarettes/d for 6 wk, lung retinol decreased. Studies are currently underway to evaluate the dose-response effect of

**TABLE 3**

Retinol concentrations of serum, lung and liver of rats exposed to cigarette smoke (SM) or air (CT) for 6 wk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum μmol/L (SEM)</th>
<th>Lung nmol/g (SEM)</th>
<th>Liver nmol/g (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>4.4 ± 0.7</td>
<td>27.9 ± 2.1</td>
<td>104.3 ± 6.6</td>
</tr>
<tr>
<td>SM</td>
<td>2.3 ± 0.3*</td>
<td>21.8 ± 1.1*</td>
<td>60.4 ± 13.0*</td>
</tr>
</tbody>
</table>

* Different from control (CT), *P < 0.05.

1 Values are mean ± SEM, n = 4, CT; n = 6, SM.
exposure to cigarette smoke on retinol and RA lung and liver concentrations.

In the present study, we found that exposure to cigarette smoke increased pulmonary inflammation and emphysema. Some potential mechanisms for the anti-inflammatory role of vitamin A include inhibition of neutrophil superoxide anion production (24), decreased release of lysosomal enzymes by neutrophils (25) and decreased conversion of arachidonic acid to the neutrophil chemoattractant leukotriene B₄ (26).

Vitamin A reduced the inflammatory response in irradiation- (27) and monocrotaline-induced lung inflammation (28) as well as in other models. In an earlier study, we deprived rats of dietary vitamin A for 6 wk and found not only increased areas of lung inflammation as we predicted, but also, unexpectedly, other areas of emphysema in the same lungs of the deficient rats (6). Our results showed that vitamin A deficiency per se induced emphysema. Our findings here and the above cited observations together suggest that the cause of increased presence of pulmonary inflammation and emphysema in cigarette smokers may be related to vitamin A status. To further support this relationship, we found that the presence and severity of emphysema was inversely related to the amount of vitamin A in the lung (Fig. 4). Additionally, some of our preliminary studies showed that dietary all-trans RA protected against the development of emphysema in cigarette-exposed rats (29).

Several mechanisms describe how vitamin A may protect against emphysema. Progressive breakdown of elastin within the alveoli is a key feature in the development of emphysema (31), thought to be caused by excessive elastase activity. Cigarette smoke inhibits resynthesis of elastin in an elastase-induced model for emphysema in hamsters (32). In contrast, vitamin A has been found to enhance the synthesis of elastin and prevent its degradation. Retinoic acid, but not retinol, increases elastin transcription (9,33–35) and elastin protein synthesis (33). This elastin induction appears to be mediated through the nuclear RA receptor-γ (RARY) as evidenced by an increased fibroblast content of RA paralleled by increased RARY mRNA (36). Furthermore, in RARY knock-out mice, there is a decrease in the elastin precursor, tropoelastin mRNA (37).

Vitamin A not only promotes elastin synthesis, but also protects against elastin degradation. Destruction of elastin may be caused by excessive neutrophil elastase activity (38) or by metalloproteinases (39). Retinoic acid inhibits the activity of human leukocyte elastase (40) and metalloproteinase activity (41). During systemic infection or inflammation, increased neutrophil elastase activity is regulated by α1-protease inhibitor (α1-PI), which contributes most of the functional antielastase protection of the alveolar wall (42). Retinol and retinaldehyde, but not RA, increased α1-protease inhibitor in corneal epithelial cells (43). Further work is necessary to determine whether these effects would be replicated in the lung.

Metaplasia or detachment of the tracheal epithelium were also detected in smoke-exposed rats in the present study. This finding was consistent with those of others (44,45) who observed metaplastic areas in cultured tracheal epithelium after exposure to cigarette smoke. In addition, vitamin A supplemented in the diet has been shown to reverse tracheal squamous metaplasia (46). Furthermore, the cigarette smoke constituent BP impairs uptake of retinol by tracheal cells (47). The detachment of tracheal epithelium may be due to decreased cell adhesion, which has been previously found in cultured tracheal epithelial cells of vitamin A–deficient hamsters (48).

Vacuolization of hepatocytes in smoke-exposed rats, as found in this study, is a typical indicator of steatosis (fatty infiltration) or multivesicular lysosomes, usually representing a toxic response. Liver injury alters vitamin A status by activating the hepatic stellate cells, the major storage site of vitamin A, and depleting its vitamin A content (49). This vacuole formation is found in a variety of liver diseases and is associated with lower hepatic vitamin A levels (50). In our previous study in which rats consumed a diet without vitamin A, we also noticed numerous vacuoles in the hepatocytes in the vitamin A–deficient rats (6).

In summary, the results of this study provide evidence that cigarette smoke induces vitamin A depletion in the serum, lung and liver, and that the compromised status of vitamin A is associated with the development of emphysema, as shown by an inverse correlation between lung vitamin A and the development of emphysema. Further studies are underway to determine whether improved vitamin A status could prevent or slow the development of emphysema in rats.

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LITERATURE CITED


FIGURE 4 Correlation between lung vitamin A concentration and emphysema evaluation score of a rat exposed to cigarette smoke for 6 wk ($r = -0.69, P < 0.03$).


