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Basic nutritional investigation

α-Tocopherol and ascorbic acid supplementation reduced acute lung inflammatory response by cigarette smoke in mouse

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Abstract

Objective: Short-term cigarette smoke (CS) exposure leads to acute lung inflammation through its influence over oxidants/antioxidants imbalance. Antioxidant vitamins such as ascorbic acid and α-tocopherol interact with oxidizing radicals. It is not clear if antioxidant supplementation can reduce inflammatory lung responses. Thus our aim was to analyze the effects of vitamin supplementation on the lungs of mice exposed to six cigarettes per day with histologic, cytological, and biochemical methods.

Methods: C57BL/6 mice were exposed to ambient air (control) or CS from 3, 6, 9, 12, or 15 cigarettes daily for up to 5 d. Mice alveolar macrophages and polymorphonuclear cells were counted in the bronchoalveolar lavage. Groups of CS animals received 50 mg/kg of ascorbic acid daily and/or 50 mg/kg of α-tocopherol daily as an oral supplementation (CS+C, CS+E, CS+C+E, respectively) 12 h before CS exposure. Thiobarbituric acid-reactive substances were detected and western blot to nuclear factor-κB were performed in lung extracts; metalloprotease-12 and tumor necrosis factor-α positive alveolar macrophages were quantified in the lungs processed for immunohistochemistry of the animals exposed to the smoke from six cigarettes daily for 5 d.

Results: The number of alveolar macrophages and polymorphonuclear cells in bronchoalveolar lavage (cells × 10^3/mL) in mice exposed to CS were increased and CS with vitamin supplementation groups presented bronchoalveolar lavage cells similar to those of control. Thiobarbituric acid-reactive substances values were reduced in vitamin supplementation groups when compared with CS and the lower value was found in the CS+C+E group. Metalloprotease-12 and tumor necrosis factor-α were more evident in CS as much as nuclear factor-κB activation when compared with control and vitamin supplementation groups.

Conclusion: Our results showed that CS induced acute lung inflammation. The inflammatory process after cigarette exposures was reduced by ascorbic acid, α-tocopherol, or more efficiently by both vitamin supplementations.

Keywords: Ascorbic acid; α-Tocopherol; Cigarette smoke; Oxidative stress; Nuclear factor-κB; Alveolar macrophage; Lung; Inflammation; Mice; Metalloprotease-12

Introduction

Chronic obstructive pulmonary disease (COPD) represents a worldwide leading cause of morbidity and mortality and it is predicted to rank as the third most common cause of death by 2020 [1,2]. Cigarette smoke (CS) is the major

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etiologic factor in the pathogenesis of COPD [3,4], and thus the majority of these cases can be prevented by quitting smoking.

Cigarette smoke contains a large number of oxidants, and it has been hypothesized that many of the adverse effects of smoking may appear as a result of oxidative damage to critical biological substances [5–7]. CS exposure increases the amount of alveolar oxidants, not only because CS itself contains an expressive number of free radicals but also because it increases the number of inflammatory cells in alveoli, which spontaneously release oxidants [8]. These oxidants inactivate α_1-antitrypsin and other protease inhibitors such as secretory leukoprotease inhibitor [5,9]. Further, the recruitment of inflammatory cells increases the protease burden, thus tipping the protease-antiprotease balance further toward the protease side [10–12]. In addition, evidence suggests that oxidative stress enhancement is associated with inflammatory cell influxes to the lung followed by lipid peroxidation and by an increase in the amount of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) [13,14]. Matrix metalloproteinases (MMPs) consist of a number of structurally related enzymes capable of digesting extracellular matrix and basement membrane components [15]. Oxidants induced by CS can directly damage components of the lung extracellular matrix such as elastin and collagen or even modify the matrix to make it more susceptible to protease attack [16]. Specific proteases derived from alveolar macrophages (AMs) and polymorphonuclear cells (PMNs) are responsible for lung injury [10–12,17]. Extracellular matrix degradation is a complex multistep process that involves a family of zinc-dependent endopeptidases known as matrix metalloproteinases. MMPs consist of a number of structurally related enzymes capable of digesting extracellular matrix and basement membrane components [15]. MMP activity is regulated at different levels, including transcriptional control, extracellular activation of proenzymes, and active enzyme inhibition [10]. The tissue inhibitors of metalloproteinases are a family with four members identified; they form complexes with active enzymes and inhibit MMP activity [18].

Vitamins are important for lung development, being involved in several reactions regarding alveoli growth and regeneration [19] and lung protection [20]. Antioxidant vitamins such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E) have been described as potent substances due to their interaction with oxidizing radicals [21]. Vitamin C is known to scavenge aqueous reactive oxygen species by rapid electron transfer and thus inhibits lipid peroxidation and reduces the level of oxidized vitamin E [22,23] and vitamin E terminates the chain reaction of lipid peroxidation in membranes and lipoproteins [23,24]. Daily oral administration of 10 mg/kg of all-trans retinoic acid in feed induced an inhibition in the growth of squamous cell carcinoma [25]. Moreover, 5 consecutive days of vitamin C or E (100 mg/kg daily) pretreatment completely prevented the formation of DNA single-strand breaks induced by CS in the lung, stomach, and liver of mice [26]. These vitamins also have beneficial effects over other mechanisms such as infectious diseases [27], and doses of 20, 30, and 50 mg/kg of food of tocopherol were equally effective in preventing aspirin-induced gastric lesions in rats [28], although it is not clear if vitamin C or E supplementations decrease lung inflammation induced by CS. After setting up an acute model of CS-induced inflammation in mice [29], our aim in this study was to analyze the effects of vitamin C and/or E supplementations on AM and PMN influxes. The following procedures were performed: Western blot to determine levels of nuclear factor-KB (NFKB), immunohistochemistry to quantify MMP-12 and TNF-α and biochemical analysis for lipid peroxidation, with the purpose of understanding the mechanisms of lung inflammation induced in this model.

Materials and methods

CS exposure

A three-step experiment was undertaken to analyze the effect of antioxidant vitamins E and C over the inflammatory cells influx into pulmonary parenchyma. All procedures were carried out in accordance with conventional guidelines for experimentation with animals and the local committee approved the experimental protocols.

Experiment 1

To study CS dose effect, 30 8-wk-old C57Bl/6 mice were exposed to 3 (3cig), 6 (6cig), 9 (9cig), 12 (12cig), or 15 (15cig) commercial filtered cigarettes per day for 5 d by using a smoking chamber (described previously) [29,30]. Mice exposed to ambient air were used as controls ($n = 5$). Each cigarette smoked produces 300 mg/m^3 of total particulate matter in the chamber.

Experiment 2

To study CS time effect, 30 8-wk-old C57Bl/6 mice were exposed to 6cig per day for 5 d to evaluate the kinetic recruitment of AMs and PMNs to the lung. Mice exposed to ambient air were used as controls ($n = 5$).

Experiment 3

To study CS and vitamin supplementation, 25 8-wk-old C57Bl/6 mice were exposed to 6cig per day for 5 d. Mice were exposed to ambient air with a basal diet without vitamin supplements (control); exposed to smoke from 6 cigarettes (CS with basal diet without vitamin supplements); exposed to smoke from 6 cigarettes plus daily supplementation with 50 mg/kg of vitamin C (CS+C); exposed to smoke from 6 cigarettes plus daily supplementation with 50 mg/kg of vitamin E (CS+E); and exposed to smoke from 6 cigarettes plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E (CS+C+E). Both vitamins were prepared daily and given for 5 d by oral

gavages 12 h before CS exposure. Vitamin C was diluted in saline solution and vitamin E was diluted in olive oil. Additional experiments were performed only with vitamin vehicles (CS plus saline, CS plus oil, and CS plus saline plus oil groups, respectively). Mice exposed to ambient air were used as controls ($n = 5$). All experiments were performed twice.

Tissue processing

One day after the last CS exposure, each mouse was sacrificed by cervical displacement and the right ventricle was perfused with saline to remove blood from the lungs. The right lung was ligated and the left lung was inflated by instilling 4% phosphate buffered formalin (pH 7.2) at 25 cm H_2O pressure for 2 min and then ligated, removed, and weighed. Inflated lungs were fixed for 48 h before embedding in paraffin. Serial sagittal 5-μm sections were obtained for morphometric and histologic analyses.

Immunohistochemistry was performed using antibodies against MMP-12 or TNF-α (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by avidin-biotin peroxidase method (goat ABC staining system, Santa Cruz Biotechnology Inc.). AMs were identified and counted in MMP-12 or TNF-α stained sections as described previously [29,30].

Bronchoalveolar lavage fluid

Airspaces were washed with buffered saline solution (300 μL) five consecutive times in the lung (final volume 1.2–1.5 mL). The fluid was withdrawn and stored on ice. Total mononuclear and polymorphonuclear cell numbers were determined in a Zi Coulter counter (Beckman Coulter, Carlsbad, CA, USA). Differential cell counts were performed on cytospin preparations (Shandon, Waltham, MA, USA) stained with Diff-Quik (Baxter Dade, Dudingen, Switzerland). At least 200 cells per bronchoalveolar lavage (BAL) fluid sample were counted using standard morphologic criteria.

Lung nuclear extracts

The right lung was minced into pieces of 2 to 4 mm with scissors and excess blood was removed by rinsing the lung pieces with saline solution. The tissue was then incubated with RPMI-1640 medium, trypsin (166 mg/mL), and ethylenediaminetetraacetic acid (EDTA; 66 mg/mL) for 30 min at 37°C. To remove particulate matter, the medium was filtered through gauze and then centrifuged for 10 min at 1500g at 4°C. The cell pellet was resuspended in medium and prepared for nuclear extracts [31]. Then pellets were lysed in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis[beta-aminoethyl ether]-N,N,N′,N′-tetraacetic acid [EGTA], 1 mM dithiothreitol, 0.5 mM phenyl methyl sulfonyl fluoride (PMSF)] and, after a 15-min incubation on ice, NP-40 was added to a final concentration of 0.5% (v/v). Nuclei were collected by centrifugation (2000g for 5 min at 4°C). The nuclear pellet was suspended in ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 20% [v/v] glycerol) and incubated for 30 min. Nuclear proteins were collected in the supernatant after centrifugation (12 000g for 10 min at 4°C) and stored in a freezer.

Immunoblotting analysis

The total protein content in the pellets from right lung tissue was determined by the method of Bradford [32]. Pellet lysates were denatured in the sample buffer (50 mM Tris-HCl, pH 6.8, 1% sodium dodecylsulfate, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heated in boiling water for 3 min. Samples (20 μg of total protein) were resolved by 15% sodium dodecylsulfate polyacrylamide gel electrophoresis and proteins transferred to polyvinyl difluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Pittsburgh, PA, USA). Rainbow markers (Amersham Pharmacia Biotech) were run in parallel to estimate molecular weights. Membranes were blocked with Tween-TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5% Tween-20) containing 2% bovine serum albumin and probed with the specific primary antibodies (Santa Cruz Biotechnology): rabbit anti-mouse actin (1:5000) and rabbit anti-mouse (p65) NFκB (1:1000). After extensive washing in Tween-TBS, the polyvinyl difluoride sheets were incubated with biotin-conjugated donkey anti-rabbit immunoglobulin G (1:1000) for 1 h and then incubated with horseradish peroxidase-conjugated streptavidin (1:1000; Caltag Laboratories, Burlingame, CA, USA). Immunoreactive proteins were visualized by 3,3′-diaminobenzidine (Sigma, Chemical Co., St. Louis, MO USA) staining. Bands were quantified by densitometry using Scion Image Software (Scion Co., Frederick, MD, USA).

Determination of oxidative stress (thiobarbituric acid-reactive substances)

The oxidative stress was measured by thiobarbituric acid-reactive substance (TBARS) concentration using a spectrophotometric method adapted from Koca et al. [33]. Samples of left lung extracts (250 μL) were deproteinized with 500 μL of 10% trichloroacetic acid and centrifuged at 900g for 10 min. The supernatant was mixed with 750 μL of 0.67% TBARS. The mixture was heated for 15 min in boiling water and then cooled. The organic phase containing the pink chromogen was extracted with 750 μL of n-butanol and absorbance at 535 nm was measured using a Beckman Spectrophotometer (model DU 640; Beckman Instruments, Fullerton, CA, USA). TBARS levels (malondialdehyde equivalents per milligram) are presented as percentages of control mice values.

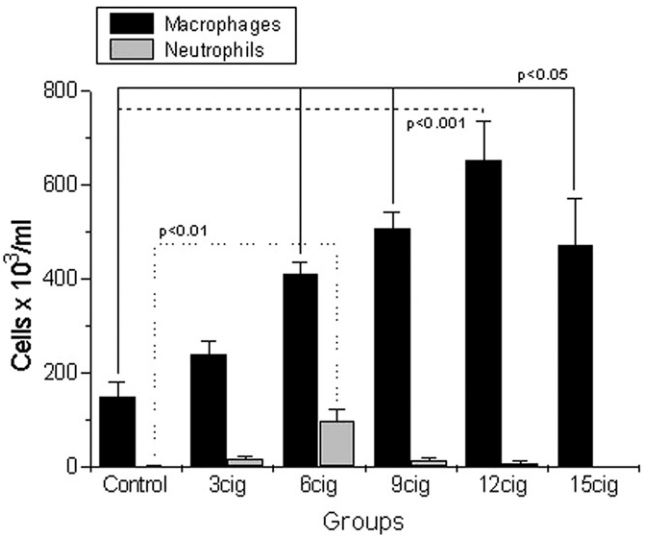

Fig. 1. Dose course of alveolar macrophage and neutrophil recruitment in the bronchoalveolar lavage of C57BL/6 mice, 1 d after the last exposure, with 3 (3cig), 6 (6cig), 9 (9cig), 12 (12cig), or 15 (15cig) three times a day for 5 d. Mice exposed to ambient air were used as a control group (Control). Data are expressed as mean ± SEM ($n = 5$; solid line, $P < 0.05$; dotted line, $P < 0.01$; dashed line, $P < 0.001$ versus control group).

Statistical analysis

Data are expressed as mean ± SEM. For comparison among groups, one-way analysis of variance was performed followed by the Student-Newman Keuls post test ($P < 0.05$). InStat Graphpad software (Graph Pad Software, San Diego, CA, USA) was used to perform the statistical analyses.

Results

Figure 1 shows the effects of cigarette doses in BAL 24 h after the last smoke exposure ($n = 5$ each for group). The number of AMs increased significantly according to cigarette doses of 6cig, 9cig, and 15cig ($P < 0.05$) and was 3.4 times more significant with 12cig ($P < 0.001$) when compared with the control group. The number of PMNs increased only in mice exposed to 6cig ($P < 0.01$) compared with the control group.

Figure 2 shows the effects of 6cig exposure from 1 to 5 d ($n = 5$ each for group). The number of AMs was more significant at days 1 and 2 ($P < 0.001$ versus control) and decreased 34% at day 5 ($P < 0.05$ at days 3, 4, and 5 versus control), whereas the number of PMNs increased 40% from days 1 to 5 ($P < 0.05$ all days versus control group).

Figure 3A shows the effects of supplementation with vitamin C, E, or both in BAL cells from mice exposed to 6cig for 5 d ($n = 5$ each for group). The supplementation with vitamin C, E, or both was found to significantly reduce AM and PMN influx after 5 d of CS exposure. Figure 3B shows the separated groups exposed to the same conditions and treated only with vehicle ($n = 5$ each for group). Saline and olive oil separately or combined did not decrease AMs and PMNs.

Histology was performed in the left lung and BAL was made in the right lung ($n = 5$ each for group). The control group exposed to ambient air presented normal-size airspaces with occasional AMs and thin alveolar septa (Fig. 4a). AMs were numerous in the lungs from the CS groups, and pulmonary tissue analysis showed PMNs; however, no changes were observed in lung histoarchitecture (Fig. 4b). The CS+C (Fig. 4c), CS+E (Fig. 4d), and CS+C+E (Fig. 4e) groups were similar to the control group, with few AMs and no or very rare PMNs. No changes were observed in elastic and collagen fibers of any group exposed to CS or ambient air.

Oxidative stress was analyzed by TBARS protocol. The control group was set up as 100% and the others groups as variation from this value ($n = 5$ each for group). Figure 5A shows the effects of supplementation with vitamin C, E, or both in TBARS detection from mice exposed to 6cig during 5 d. Supplementation with vitamin C reduced TBARS level ($P < 0.05$), whereas supplementation with vitamin E ($P < 0.01$) or both ($P < 0.001$) was more efficient. Figure 5B shows the separated groups exposed to previously cited conditions and treated only with vehicle. Saline and olive oil separately or combined had no effect on TBARS levels.

Alveolar macrophages were counted in 10 different counting fields per animal section from lung tissue immunostained for MMP-12 or TNF-α (Figs. 6, 7, and 8). The number of AMs per square millimeter expressing MMP-12 and TNF-α increased in the lungs of the CS group ($P <$

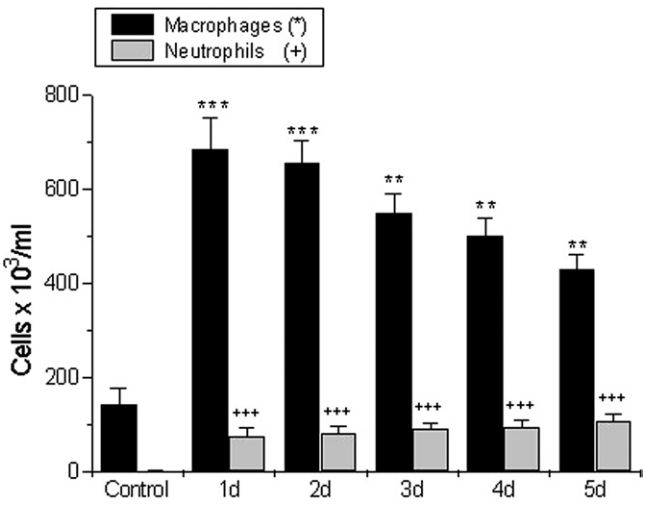

Fig. 2. Time course of alveolar macrophages and neutrophil recruitment in the BAL of C57BL/6 mice after cigarette smoke exposure with 6 cigarettes per day for 1 d (1d), 2 d (2d), 3 d (3d), 4 d (4d), or 5 d (5d). Mice exposed to ambient air were used as a control group (Control). Data are expressed as mean ± SEM ($n = 5$; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{+++}P < 0.001$; *to macrophages and $^{+}$to neutrophils versus control group from each one). NFκB, nuclear factor-κB.

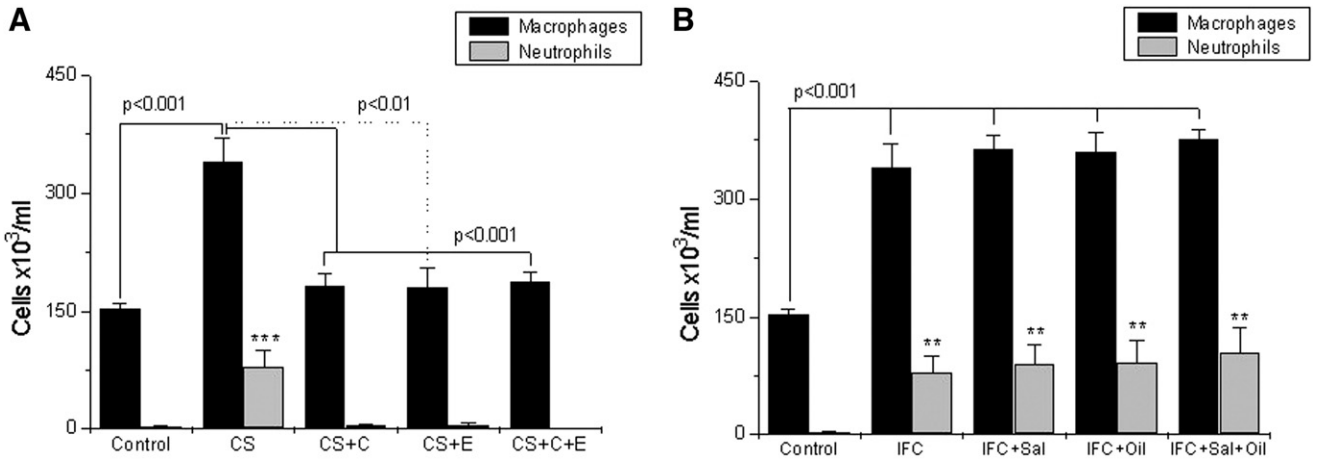

Fig. 3. Effects of vitamin supplementation on alveolar macrophage and neutrophil recruitment in the bronchoalveolar lavage of C57BL/6 mice after cigarette smoke exposure with 6 cigarettes per day for 5 d. Data are expressed as mean ± SEM ($n = 5$; solid line, $P < 0.001$; dotted line, $P < 0.01$; $^{***}P < 0.001$; lines to macrophages and *to neutrophils versus control group). (a) The groups consisted of mice exposed to cigarette smoke (CS), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C (CS+C), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin E (CS+E), or cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E (CS+C+E). Mice exposed to ambient air were used as a control group (Control). (b) The groups consisted of mice exposed to cigarette smoke (CS), cigarette smoke plus saline (CS+sal), cigarette smoke plus olive oil (CS+oil), or cigarette smoke plus saline plus olive oil (CS+sal+oil). Mice exposed to ambient air were used as a control group (Control).

0.001), whereas the number in CS+C, CS+E, and CS+C+E, were decreased when compared with the control group. The percentage of AMs expressing MMP-12 was minor compared with AMs expressing TNF-α in the lungs of CS mice, whereas the percentage in CS+C, CS+E, and CS+C+E mice was similar to that in the control group for MMP-12 and TNF-α, except CS+C for MMP-12 (Fig. 8). Supplementation with vitamin C, E, or both also reduced activation of NFκB (confirmed by western blotting) as shown in Figure 9. The CS group exhibited an evident activation of NFκB, whereas the control group had a weak band as demonstrated by densitometric analysis (Fig. 10).

Discussion

Cigarette smoke has been implicated as the major risk factor for the development of COPD [7,12]. The adverse action of the CS is due to the presence of a large variety of compounds such as nicotine, benzo(a)pyrene, oxidants, and free radicals that could initiate, promote, and/or amplify oxidative damage [34,35]. CS is the major determinant of diseases related to oxidative stress and the individual variations in the incidence and extension of COPD are numerous [36]. Specific reasons such as genetic susceptibility, extension of exposure to CS, and/or presence of certain antioxidant micronutrients such as vitamins C and E in the daily diet of humans or animals could be attributed to these variations [7,37,38].

First, mice were exposed to different doses of CS for 5 d to determine inflammatory cell recruitment to the lungs. CS exposure induced AM and PMN influxes differently. A similar result reported by Castro et al. [39], with differences in the exposure model, shows the critical dose response in different strains. The CS dose exposure and the genetic background relation with the macrophage and PMN alveolar influxes may explain why only 15% to 20% of smokers develop emphysema. This indicates how cigarette dose may play a critical role in the development of COPD. We also analyzed the kinetic of AM and PMN influxes with 6cig exposure. Macrophage and PMN counts in BAL were distinct from those in controls and, although PMN exhibited an increase from day 1 to day 5, no significant differences were detected; on the contrary, a decrease in the number of AMs was observed from day 1 to day 5 of CS exposure.

We tested dietary antioxidant (vitamins C and E) supplementation in mice exposed to 6cig for 5 d. We showed that vitamin C, vitamin E, or both were able to reduce AM and PMN influxes. Oxidative stress may be increased because of the high concentration of oxidants in CS, the production of oxidants by activated inflammatory cells, and a reduction in endogenous antioxidant mechanisms [16]. Malondialdehyde, which is a local and systemic marker of oxidative stress, is increased particularly during exacerbations in COPD [23, 40]. Malondialdehyde levels have been used as a convenient index of the lipid peroxidation-related oxidative damage of tissues of smokers [41, 42], TBARS are also markers of lipid peroxidation and were increased in exhaled breath condensate of patients with COPD [43, 44]. Although TBARS were usually associated with other parameters for evaluation of lipid peroxidation and oxidative stress [45–47], in this study vitamin supplementation groups showed decreased levels of TBARS (similar to control group) when compared with the CS group.

In this study, we observed that vitamin supplementation is efficient in reducing MMP-12 detection on AMs. Hauta-

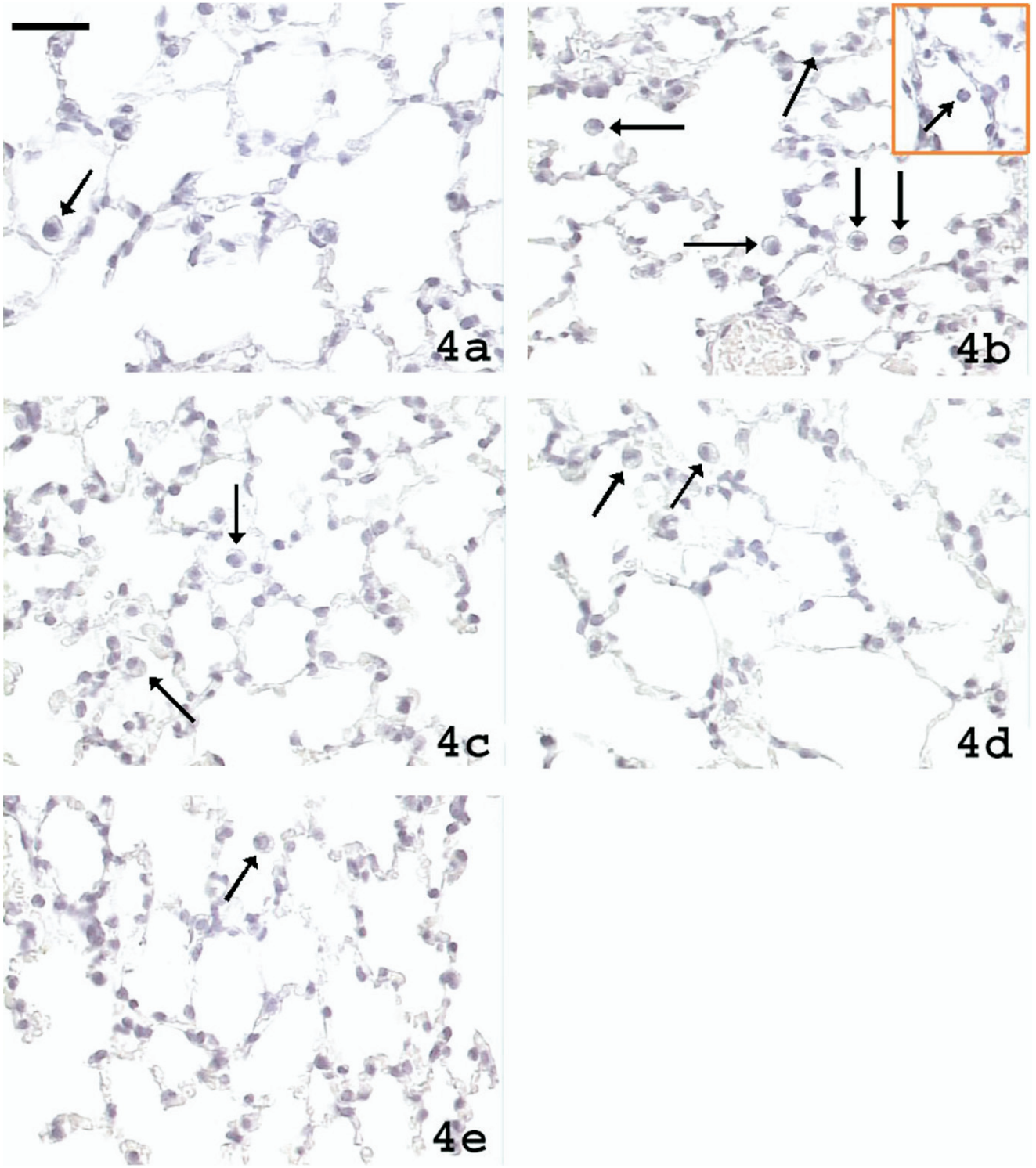

Fig. 4. Photomicrographs of lung sections stained with hematoxylin and eosin (bar = 50 μm). (a) Control group (mice exposed to ambient air). Lung parenchyma exhibit normal-size airspaces and thin alveolar septa with occasional alveolar macrophages (black arrows). (b) CS group (mice exposed to six cigarettes per day for 5 d). Normal-size airspaces and thin alveolar septa with many alveolar macrophages (black arrows) are seen. Into the red line a neutrophil can be visualized (black arrow). (c) CS+C group (mice exposed to six cigarettes per day for 5 d plus daily supplementation with 50 mg/kg of vitamin C). The histologic pattern is similar to that of the control group. (d) CS+E group (mice exposed to six cigarettes per day for 5 d plus daily supplementation with 50 mg/kg of vitamin E). The histologic pattern is similar to that of the control group. (e) CS+C+E group (mice exposed to six cigarettes per day for 5 d plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E). The histologic pattern is similar to that of the control group.

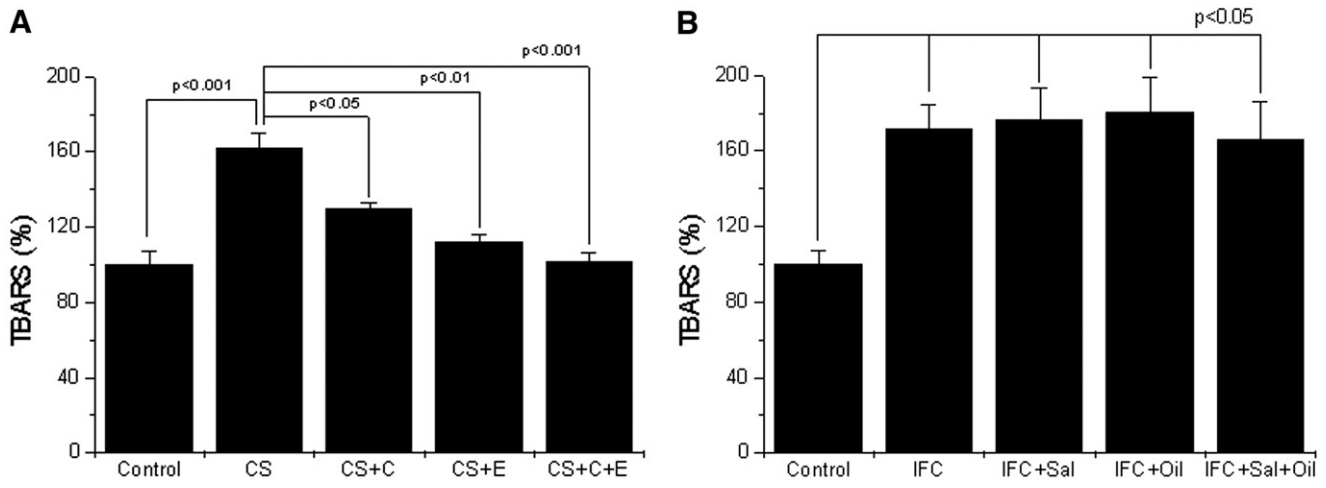

Fig. 5. Effects of vitamin supplementation on TBARS (%) in the left lung extracts of C57BL/6 mice after cigarette smoke exposure with six cigarettes per day for 5 d. Data are expressed as mean ± SEM ($n = 5$; solid line, $P < 0.001$, $P < 0.01$, or $P < 0.05$ versus CS group). (a) The groups consisted of mice that were exposed to cigarette smoke (CS), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C (CS+C), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin E (CS+E), or cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E (CS+C+E). Mice exposed to ambient air were used as a control group (Control). (b) The groups consisted of mice exposed to cigarette smoke (CS), cigarette smoke plus saline (CS+sal), cigarette smoke plus olive oil (CS+oil), or cigarette smoke plus saline plus olive oil (CS+sal+oil). Mice exposed to ambient air were used as a control group (Control). Data are expressed as mean ± SEM ($n = 5$; solid line, $P < 0.05$ versus control group). TBARS, thiobarbituric acid-reactive substances.

maki et al. [48] reported that mice lacking MMP-12 were completely protected against CS-induced emphysema. MMP-12 must play the same role in humans [49]. We suggest that vitamin supplementation by reducing MMP-12 production may abrogate the development of emphysema, at least in mice, because similar results could be found in long-term CS exposure.

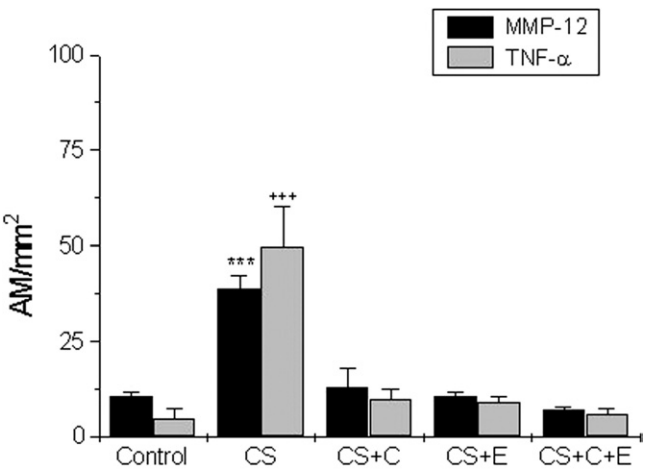

Fig. 6. Effects of vitamin supplementation evaluated by morphometry on AMs positive for MMP-12 or TNF-α per area (square millimeters). The groups consisted of mice that were exposed to cigarette smoke (CS), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C (CS+C), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin E (CS+E), or cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E (CS+C+E). Mice exposed to ambient air were used as a control group (Control). Data are expressed as mean ± SEM ($n = 5$; $^{***}P < 0.001$ to MMP-12 and $^{+++}P < 0.001$ to TNF-α versus control group from each one). AM, alveolar macrophage; MMP-12, matrix metalloproteinase-12; TNF-α, tumor necrosis factor-α.

Tumor necrosis factor-α is a powerful cytokine, as a key mediator of inflammation, and also plays an important role in host defense. TNF-α activates macrophages and epithelial cells to produce various inflammatory cell chemoattractants [11]. MMPs have TNF-α converting activity, and we performed morphometry in AMs expressing TNF-α. Vitamin supplementation was able to reduce TNF-α detection in AMs compared with the CS group. These findings lead us to propose a model in which smoke induces AMs to secrete MMP-12, which then releases TNF-α, activating endothelial cells and leading to constant inflammatory cells influx.

A limitation of the present study was the relatively short duration of CS exposition. A study of long-term exposure to CS is necessary to understand possible vitamin actions in reducing COPD development. According to our results in previous studies [10–12,29], the AM could be the key inflammatory cell in the mouse model of CS. Although PMNs respond at an exposure of 6cig, no other exposure doses showed any significant changes. There was no dose response for PMNs. Therefore, the role of PMNs in smoke-induced inflammation and injury, according to the present data, is not clear. However, It is becoming increasingly evident that other mechanisms, in addition to matrix proteolysis induced by neutrophil elastase and other matrix proteases, are implicated in the development of emphysema, such as oxidative stress and apoptosis of alveolar septal cells [17,50].

The induction of inflammatory mediators can be regulated by the activation of redox-sensitive transcription fac-

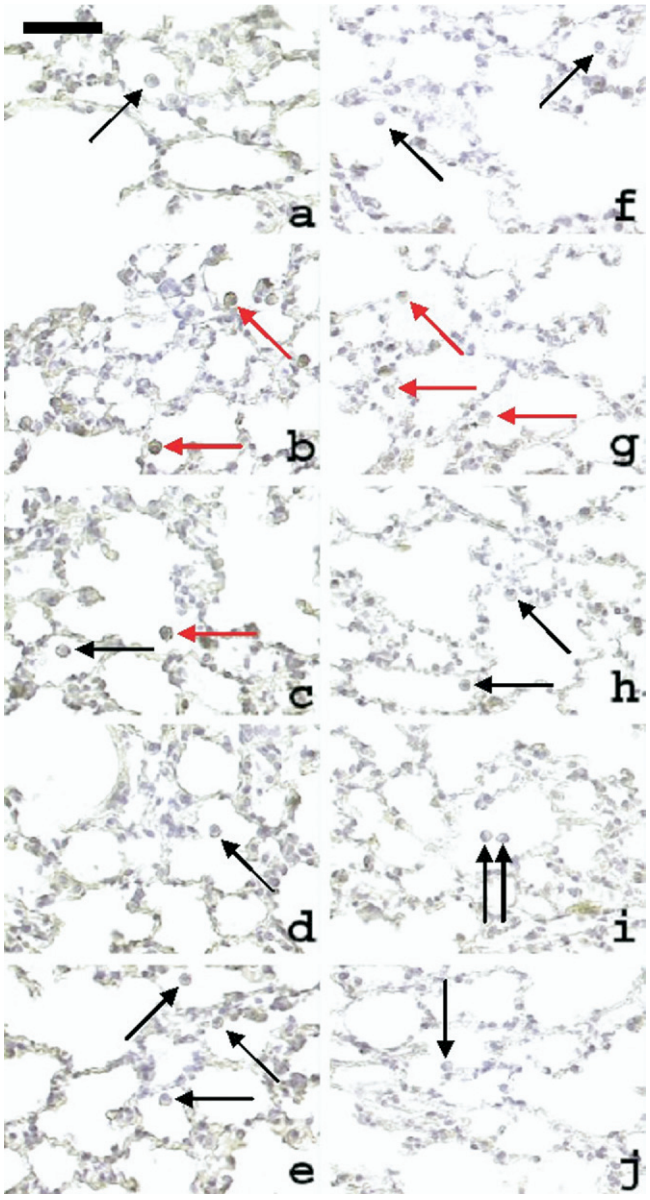

Fig. 7. Immunohistochemistry sections from all groups for matrix metalloproteinase-12 (left column) and tumor necrosis factor-α (right column). Bar = 100 μm. Red arrows indicate positive macrophages, and black arrows indicate non-positive cells. (a, f) Mice exposed to ambient air were used as a control group. Positive alveolar macrophages were reduced in the vitamin supplementation groups when compared with the CS group. The groups consisted of mice that were exposed to (b, g) cigarette smoke (CS), (c, h) cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C (CS+C), (d, i) cigarette smoke plus daily supplementation with 50 mg/kg of vitamin E (CS+E), or (e, j) cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E (CS+C+E).

tors such as NFκB. Activation of NFκB in response to oxidants and inflammatory cytokines, such as TNF-α and interleukin-6 released during the inflammation process, is important in the recruitment and activation of inflammatory cells [51]. In our study, we showed that vitamin supplementation reduced AM and PMN influxes, probably by a direct

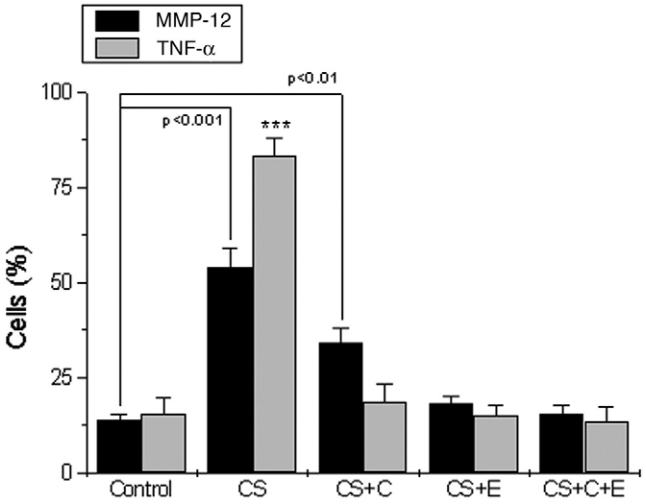

Fig. 8. Effects of vitamin supplementation evaluated by morphometry on alveolar macrophages positive for matrix metalloproteinase-12 or tumor necrosis factor-α/100. The groups consisted of mice that were exposed to cigarette smoke (CS), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C (CS+C), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin E (CS+E), or cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E (CS+C+E). Mice exposed to ambient air were used as a control group (Control). Data are expressed as mean ± SEM (n = 5; $^{***}P < 0.001$ to TNF-α versus control group).

action on NFκB activation. NFκB was detected in the CS group; supplementation with vitamins decreased activation of NFκB, with bands similar to those in the control group.

Conclusion

Alveolar macrophages, NFκB, MMP-12, and TNF-α significantly increase in response to CS exposure. The antiox-

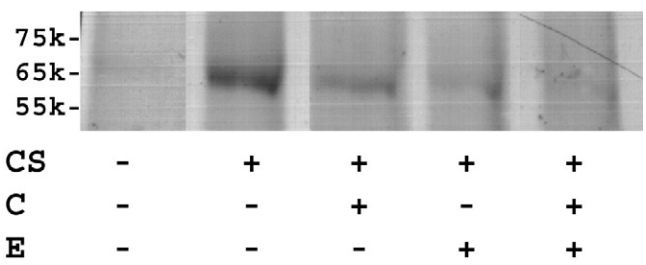

Fig. 9. Effects of vitamin supplementation on western blotting to nuclear factor-κB of left lung extracts in C57BL/6 mice after exposure to cigarette smoke from six cigarettes per day for 5 d. Negative signal (−) indicates ambient air exposure and/or no vitamin supplementation. Positive signal (+) indicates cigarette smoke exposure and/or vitamin supplementation. The vitamin supplementation groups showed decreased nuclear factor-κB activation when compared with the CS group. The groups consisted of mice that were exposed to cigarette smoke (CS), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C (CS+C), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin E (CS+E), or cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E (CS+C+E). Mice exposed to ambient air were used as a control group (Control).

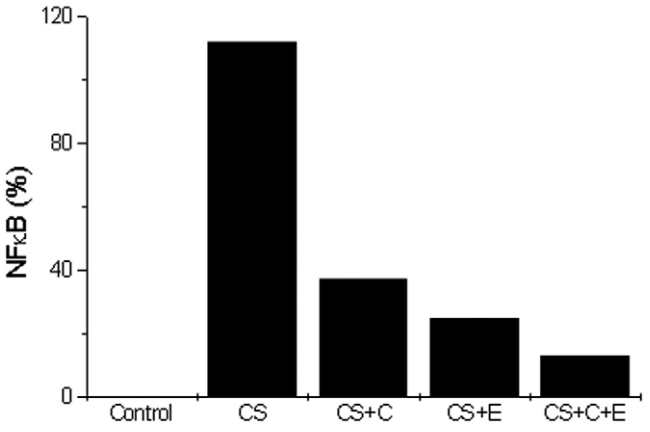

Fig. 10. Effects of vitamin supplementation on western blotting to nuclear factor-κB of left lung extracts in C57BL/6 mice after exposure to cigarette smoke from six cigarettes per day for 5 d. Blots were analyzed by densitometry and the content of nuclear factor-κB in nuclear extracts was expressed as a percentage of that in the control group. Results are representative of two similar experiments. The groups consisted of mice that were exposed to cigarette smoke (CS), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C (CS+C), cigarette smoke plus daily supplementation with 50 mg/kg of vitamin E (CS+E), or cigarette smoke plus daily supplementation with 50 mg/kg of vitamin C and 50 mg/kg of vitamin E (CS+C+E). Mice exposed to ambient air were used as a control group (Control).

idant nutrients vitamins C and E prevent this response to some degree. The inflammatory process after cigarette exposures was reduced by ascorbic acid, α-tocopherol, or more efficiently by both vitamin supplementations.

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