

Role of Oxidative Stress in Ultrafine Particle–induced Exacerbation of Allergic Lung Inflammation

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Rationale: The effects of ultrafine particle inhalation on allergic airway inflammation are of growing interest. The mechanisms underlying these effects are currently under investigation.

Objectives: To investigate the role of oxidative stress on the adjuvant activity of inhaled elemental carbon ultrafine particles (EC-UFPs) on allergic airway inflammation.

Methods: Ovalbumin-sensitized mice were exposed to EC-UFPs (504 $\mu\text{g}/\text{m}^3$ for 24 h) or filtered air immediately before allergen challenge and systemically treated with N-acetylcysteine or vehicle before and during EC-UFP inhalation. Allergic inflammation was measured up to 1 week after allergen challenge by means of bronchoalveolar lavage, cytokine/total protein assays, lung function, and histology. Isoprostane levels in lung tissue served to measure oxidative stress. Transmission electron microscopy served to localize EC-UFPs in lung tissue and both electrophoretic mobility shift assay and immunohistochemistry to quantify/localize nuclear factor- κB (NF- κB) activation.

Measurements and Main Results: In sensitized and challenged mice EC-UFP inhalation increased allergen-induced lung lipid peroxidation and NF- κB activation in addition to inflammatory infiltrate, cytokine release, and airway hyperresponsiveness. Prominent NF- κB activation was observed in the same cell types in which EC-UFPs were detected. N-acetylcysteine treatment significantly reduced the adjuvant activity of EC-UFPs. In nonsensitized or sensitized but not challenged mice EC-UFP exposure induced a moderate increase in isoprostanes but no significant effect on other parameters of lung inflammation.

Conclusions: Our findings demonstrate a critical role for oxidative stress in EC-UFP-induced augmentation of allergen-induced lung inflammation, where EC-UFP exposure has potentiating effects in lung allergic inflammation. Our data support the concept that allergic individuals are more susceptible to the adverse health effects of EC-UFPs.

Keywords: air pollution; particulate matter; allergy; oxidative stress

Numerous studies have reported associations between exposure to particulate matter (PM) and adverse health effects, including increases in respiratory morbidity and enhanced mortality rates (1–5). Ultrafine particles (UFPs, $<0.1 \mu\text{m}$ in thermodynamic diameter) represent a fraction of fine particulate matter (aero-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The carbonaceous core of environmental fine particulates has been shown to have adjuvant activity in allergic diseases. The underlying mechanisms are still unclear.

What This Study Adds to the Field

Ultrafine carbon particle inhalation augments allergen-induced lipid peroxidation and nuclear factor- κB activation in addition to lung inflammation, cytokine release, and airway hyperactivity. Oxidative stress is central to this process.

dynamic diameter $<2.5 \mu\text{m}$ [$\text{PM}_{2.5}$]) of growing interest for their central role in causing respiratory health effects (4, 6–9). UFPs are characterized by a low mass concentration but high number concentration and a large surface area. Primary UFPs are formed during gas-to-particle conversion or during incomplete fuel combustion processes (10). Compared with larger particles, they have a higher deposition rate in the peripheral lung, can cross the alveolar epithelium and reach the interstitium, and have enhanced capabilities to produce reactive oxygen species (ROS) (11–14). Their role as adjuvant both in the sensitization phase and in the elicitation phase of the allergic response has well been documented (15–18).

Free radicals and oxidative stress are important contributors to lung inflammation, including lung allergic inflammation (19, 20). Pulmonary exposure to UFPs is also known to cause oxidative stress, which increases ROS production (13, 14, 21). Enhanced levels of ROS have been well documented to induce or to augment the activation of the redox-sensitive transcription factors nuclear factor- κB (NF- κB) in the lung, which triggers the release of inflammatory mediators (22, 23). Moreover, antioxidant treatment has been shown to interfere with the generation of oxidative stress and to impede the development of various respiratory diseases (24). In particular, thiol antioxidants, such as N-acetylcysteine (NAC) and N-acetylcystine (lysine salt of NAC) have been shown, in *in vitro* and *in vivo*, to block the release of pulmonary inflammatory mediators by acting as radical scavenger, increasing intracellular glutathione level, and decreasing NF- κB activation (22, 25–28). In addition, they have been shown to be effective in reversing the adjuvant effects of diesel exhaust particles during the sensitization phase of the allergic response (29).

We have previously shown that exposure to elemental carbon (EC)–UFPs alone exerts a strong adjuvant effect on the manifestation of allergic airway inflammation (18). In the

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present study we sought to investigate the role of oxidative stress in the UFP-induced augmentation of allergic lung inflammation. We compared physiological effects of particle exposure in allergic and normal control mice with and without the addition of a thiol antioxidant, N-acetylcysteine. We evaluated bronchoalveolar lavage (BAL) cellular composition and cytokines in BAL fluid, performed lung function tests, measured oxidative stress by quantifying 8-isoprostane in lung homogenates, and measured and localized NF- κ B activation and UFP localization in pulmonary cells.

METHODS

Animals and Materials

Five- to 7-week-old female Balb/c mice (Charles River, Sulzfeld, Germany) were housed in individually ventilated cages (VentiRack, cage type CU-31; BioZone, Kent, UK) and received a standard pellet diet and water *ad libitum*. The study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria. All chemicals were purchased from Sigma-Aldrich Chemie, Deisenhofen, Germany, unless otherwise specified.

Allergen Sensitization/Challenge Protocol

To evaluate the adjuvant effects of EC-UFP inhalation, a protocol of mild allergic inflammation of the lung was used. Details of sensitization and challenge protocols are provided in the online supplement. Ovalbumin (OVA)-specific IgE and IgG1 were measured as described previously (18). OVA/alum-sensitized mice, compared with nonsensitized control mice, were characterized by high titers of OVA-specific IgE (31.7 ± 3.3 vs. 0.1 ± 0.01 μ g/ml) and OVA-specific IgG1 ($1,178.4 \pm 211.2$ vs. <0.1 μ g/ml). A 20-minute OVA/phosphate-buffered saline (PBS) aerosol challenge was performed by Pari-Boy nebulization.

Elemental Carbon Ultrafine Particle Exposure

EC-UFP production, characterization, and details of animal exposures are provided in the online supplement. All EC-UFP exposures used for this study were performed for 24 hours with a mass concentration of $504 \mu\text{g}/\text{m}^3$, which corresponds to an average particle number concentration of $8.8 \times 10^6/\text{cm}^3$.

Study Design

A schematic representation of the study protocol is shown in Figure 1.

To evaluate the effect of EC-UFP-induced oxidative stress on the allergic inflammation of the lung, sensitized (S/UFP) and sensitized and subsequently challenged (S/UFP/OVA) mice were exposed to EC-UFPs with and without NAC treatment. NAC was administered with two intraperitoneal injections (250 mg/kg body weight in PBS, 200 μ l), once shortly before and once close to mid-EC-UFP exposure. OVA-sensitized and subsequently challenged mice exposed to filtered air (S/OVA) and nonsensitized mice exposed to EC-UFPs (NS/UFP) with and without NAC treatment served as controls. In addition, untreated animals served as baseline controls ($n = 12$). BAL was performed at 0, 24, or 168 hours after OVA challenge, or after termination of EC-UFP exposure ($n = 4$ per time point). Lung function tests (*, Figure 1) were performed: (1) by body plethysmography in untreated animals ($n = 12$), 24 hours after EC-UFP inhalation in NS/UFP ($\pm$ NAC) and S/UFP or after OVA challenge in S/OVA and in S/UFP/OVA ($\pm$ NAC) ($n = 5$ per group); (2) in intubated/mechanically ventilated animals 24 hours after OVA challenge in S/OVA, S/UFP/OVA, S/NAC/UFP/OVA, and in untreated mice ($n \geq 4$ per group).

The description of the methods used for BAL, measurement of total protein and cytokines in BAL fluid, histological and immunohistochemical evaluations, transmission electron microscopy, and measurements of airway hyperresponsiveness, 8-isoprostane, and NF- κ B activation in lung homogenates is provided in the online supplement.

Statistical Analysis

Data were expressed as mean $\pm$ SD. In lung function tests data were expressed as mean $\pm$ SEM. For statistical evaluation, one-way analysis

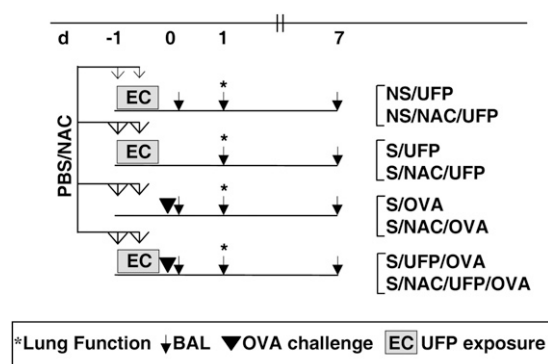


Figure 1. Experimental groups (left) and designation (right): EC = 24-hour exposure to elemental carbon ultrafine particles (EC-UFPs) ($504 \mu\text{g}/\text{m}^3$); NAC = two intraperitoneal injections with N-acetylcysteine; ▼ = ovalbumin (OVA) aerosol challenge; ↓ = sacrifice, bronchoalveolar lavage (BAL), and histology; * = lung function tests. NS = nonsensitized; OVA = challenged with aerosolized OVA; S = sensitized; UFP = EC-UFP exposed.

of variance with post-hoc Scheffé test (applied for BAL cells and BAL fluid total protein and cytokines analysis) and least significant difference test (LSD) (applied for lung function tests and isoprostane concentration) comparisons were used (Statistica 6.0; StatSoft, Inc., Tulsa, OK). A *P* value less than 0.05 was considered to be significant.

RESULTS

NAC Effectively Reduces EC-UFP-dependent Augmentation of Allergen-induced Airway Inflammation

In sensitized mice, EC-UFP inhalation before OVA challenge (S/UFP/OVA) caused a significant increase of BAL inflammatory infiltrate 7 days after OVA challenge, compared with sensitized and challenged mice (S/OVA) exposed to clean air (Figure 2A). The enhanced response was significantly reduced by NAC treatment (S/NAC/UFP/OVA). NAC reduced moderately but not significantly the BAL inflammatory infiltrate of S/OVA mice at the 7-day time point (S/NAC/OVA, Figure 2A). In the absence of OVA challenge, mice exposed to EC-UFPs did not develop a significant BAL inflammatory infiltrate regardless of whether they had been sensitized or not (NS/UFP, Figure 2A; S/UFP, data not shown). NAC had no detectable effect on the BAL inflammatory infiltrate of NS/UFP and S/UFP mice (data not shown).

A similar pattern emerged when protein and cytokine concentrations were analyzed in BAL fluid. In sensitized mice, OVA challenge alone caused an increase in BAL fluid total protein. A further increase was detected in sensitized mice exposed to EC-UFP inhalation before OVA challenge (S/UFP/OVA) at the 7-day time point (Figure 2B). NAC reduced BAL fluid total protein concentrations (S/NAC/UFP/OVA), but not significantly. In sensitized mice, EC-UFP inhalation before OVA challenge (S/UFP/OVA) caused a significant increase in IL-13 and IL-5 when compared with mice exposed to filtered air (S/OVA) (Figure 2B). NAC abolished the adjuvant effect of EC-UFPs for both BAL fluid cytokines (Figure 2B). No significant alterations in IL-10 and TGF- β concentrations were observed in any of the groups (data not shown). No induction of cytokines and protein levels were observed in any of the nonchallenged groups (NS/UFP, S/UFP, with and without NAC treatment, NS/UFP shown in Figure 2B).

NAC Reduces EC-UFP Increase in Inflammatory Infiltrate

Lung histological analysis was focused on the 7-day time point because this time point was more representative for inflammatory

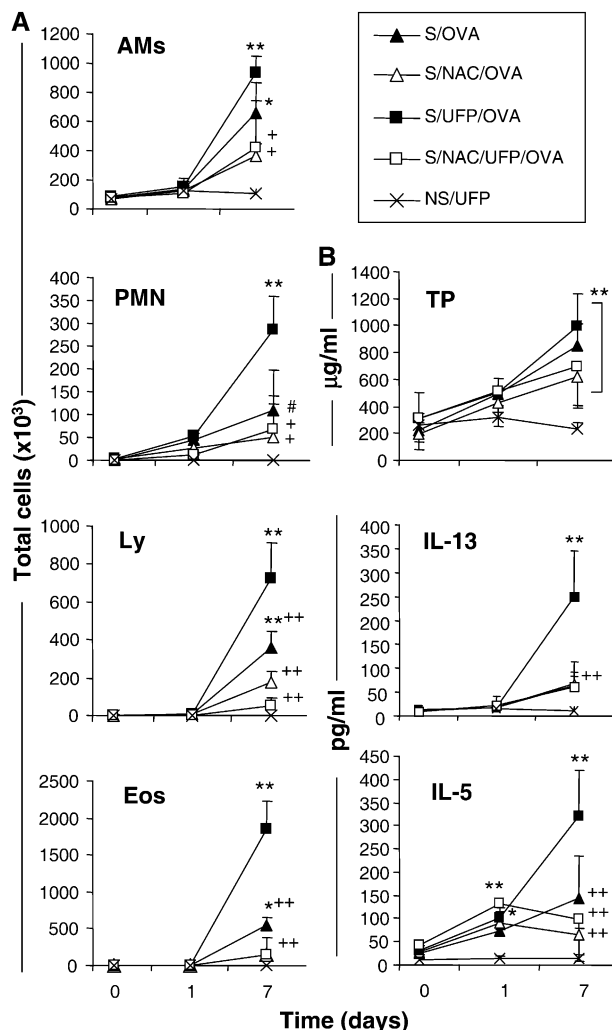


Figure 2. Bronchoalveolar lavage (BAL) cells and cytokines. (A) BAL cells and (B) BAL fluid total protein (TP) and cytokines in sensitized and challenged mice (S/OVA), treated with N-acetylcysteine (NAC) (S/NAC/OVA), or exposed to elemental carbon ultrafine particles (EC-UFPs) 24 hours before ovalbumin (OVA) challenge (S/UFP/OVA), and treated with NAC before and close to mid-EC-UFP exposure (S/NAC/UFP/OVA). Nonsensitized mice exposed to EC-UFP (NS/UFP) served as controls. Data presented as mean $\pm$ SD ($n = 4$ per time point for BAL cells and $n = 4-8$ per time point for TP and cytokines). AMs = alveolar macrophages; Eos = eosinophils; Ly = lymphocytes; PMN = polymorphonuclear neutrophils. * $P < 0.05$, ** $P < 0.01$ versus NS/UFP; + $P < 0.05$, ++ $P < 0.01$, # $P = 0.05$ versus S/UFP/OVA.

alterations and goblet cell hyperplasia. Seven days after UFP exposure, no alterations in the lungs of nonsensitized (NS/UFP) and sensitized (S/UFP) mice exposed to EC-UFPs (Figure 3A, NS/UFP) were detected. S/OVA showed the characteristic infiltrate and goblet cell hyperplasia (Figure 3B). Sensitized and challenged mice exposed to EC-UFPs showed strongest inflammatory infiltrate and goblet cell hyperplasia (S/UFP/OVA, Figure 3C). These histological alterations were strongly reduced by NAC treatment (S/NAC/UFP/OVA, Figure 3D).

EC-UFP-induced Lipid Peroxidation Can Be Reversed by NAC Treatment

To evaluate the role of oxidative stress in EC-UFP-induced increase in allergic inflammation of the lung, 8-isoprostane

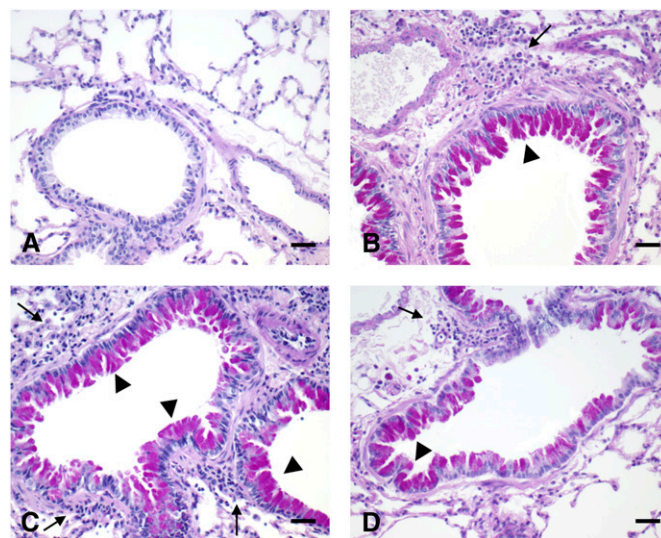


Figure 3. Lung histology 7 days after elemental carbon ultrafine particle (EC-UFP) exposure/ovalbumin (OVA) challenge (periodic acid-Schiff staining) in representative samples. (A) Nonsensitized mice exposed for 24 hours to EC-UFPs (NS/UFP); (B) sensitized and challenged mice exposed to filtered air (S/OVA); (C) sensitized mice exposed to EC-UFP 24 hours before OVA challenge (S/UFP/OVA); (D) sensitized mice exposed to EC-UFP 24 hours before OVA challenge and treated with NAC (S/NAC/UFP/OVA). Arrows, inflammatory infiltrate; arrowheads, mucus hypersecretion; scale bar, 32 μ m.

levels were analyzed in lung homogenates of all experimental groups. The concentration of 8-isoprostane in untreated lungs was 845 ± 72 pg/mg protein (Figure 4A, dotted line). Although EC-UFP exposure in mice that were not challenged did not induce an increase in inflammatory infiltrate (NS/UFP, Figures 2 and 3; S/UFP, data not shown), it induced a moderate but significant increase in 8-isoprostane concentration (NS/UFP, S/UFP $P < 0.05$, $P < 0.01$ vs. untreated). NAC treatment before and during EC-UFP exposure reduced the particle-induced lipid peroxidation (Figure 4A).

In sensitized mice, allergen challenge induced increased levels of 8-isoprostane (S/OVA, Figure 4B, ** $P < 0.01$ vs. untreated). NAC treatment reduced the allergen-induced lipid peroxidation almost back to levels of untreated control mice (S/NAC/OVA, not significantly different from untreated). EC-UFP inhalation before OVA challenge further augmented the lipid peroxidation (S/UFP/OVA, ** $P < 0.01$ vs. untreated; # $P < 0.05$ vs. S/OVA), an effect that was reversed by NAC treatment (S/NAC/UFP/OVA) almost completely at day 1 (** $P < 0.01$ vs. S/UFP/OVA) and still partially at day 7 after allergen challenge (+ $P = 0.05$ vs. S/UFP/OVA).

NAC Reduces UFP-dependent Increase in Airway Hyperresponsiveness

Lung function tests performed 24 hours after OVA challenge showed significantly increased enhanced pause (Penh) and respiratory system resistance (R) after increasing methacholine concentrations in OVA-sensitized animals exposed to EC-UFPs before OVA challenge (S/UFP/OVA) compared with untreated (S/UFP/OVA vs. untreated: * $P < 0.05$, ** $P < 0.01$, Figures 5A and 5B) and compared with OVA-sensitized and -challenged animals (S/UFP/OVA vs. S/OVA: § $P < 0.05$, Figure 5B). S/OVA animals showed significantly increased R compared with untreated. Penh was also increased in S/OVA animals, but this did not reach statistical significance. NAC significantly reduced

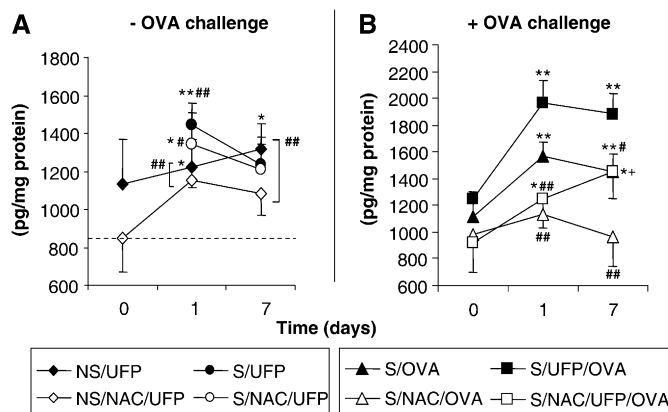


Figure 4. Isoprostane concentration. (A) Isoprostane concentration in nonsensitized and sensitized mice exposed to elemental carbon ultrafine particles (EC-UFPs) for 24 hours (NS/UFP, S/UFP, respectively), and treated with N-acetylcysteine (NAC) (NS/NAC/UFP, S/NAC/UFP, respectively). (B) Sensitized and challenged mice exposed to filtered air (S/OVA), and treated with NAC (S/NAC/OVA) or exposed to EC-UFPs (S/UFP/OVA) and treated with NAC (S/NAC/UFP/OVA). The level in untreated mice is shown by a dotted line. NAC was always given as two intraperitoneal injections before and close to mid-EC-UFP (or filtered air) exposure. Mice were killed at time 0, 1, or 7 days after EC-UFP (or filtered air) exposure. Data are expressed as mean $\pm$ SEM ($n = 10$ for untreated lungs; $n = 4-9$ per time point per group). * $P < 0.05$, ** $P < 0.01$ versus untreated; # $P < 0.05$, ## $P < 0.01$, + $P = 0.05$ versus S/UFP/OVA.

UFP-dependent increase in airway responsiveness (S/UFP/OVA vs. S/NAC/UFP/OVA: + $P < 0.05$, ++ $P < 0.01$, Figures 5A and 5B). In the absence of allergen challenge, lung function tests in nonsensitized (NS/UFP) or sensitized (S/UFP) mice exposed to EC-UFPs with or without NAC treatment were similar to the ones in untreated animals (data not shown).

Enhanced NF- κ B Activation in EC-UFP-exposed and OVA-challenged Lungs Is Inhibited by NAC

To investigate the potential involvement of NF- κ B in mediating EC-UFP-increased inflammatory responses in mouse lung tissue of sensitized and challenged mice exposed to EC-UFPs compared with those exposed to filtered air, NF- κ B activation was analyzed at the 7-day time point in lung homogenates of S/UFP/OVA and S/OVA. In addition, non-challenged groups (NS/UFP and S/UFP) and untreated control mice were analyzed. Clearly, UFP treatment strongly augmented NF- κ B activation in the lungs of OVA-sensitized and -challenged mice (S/UFP/OVA, Figures 6A and 6B). No significant activation of NF- κ B was found in lungs of non-sensitized or sensitized mice exposed to EC-UFPs at the 7-day time point (Figures 6A and 6B). To verify the ability of EC-UFPs alone in inducing NF- κ B activation at earlier time points, lung homogenates from nonsensitized mice exposed to EC-UFPs (with or without NAC treatment) and relative control mice (untreated) were analyzed for NF- κ B activation immediately after the termination of EC-UFP exposure (time 0). A moderate NF- κ B activation was found in the lungs of NS/UFP compared with untreated mice, which was reduced by NAC treatment (see Figure E1 in the online supplement). To verify NF- κ B DNA-binding activity and to determine NF- κ B subunit composition, we performed a supershift analysis of two S/UFP/OVA lung samples using antibodies against the p50 or p65 subunit of NF- κ B (Figure 6C). NF- κ B binding activity was composed of p50/p65 heterodimers, which are the

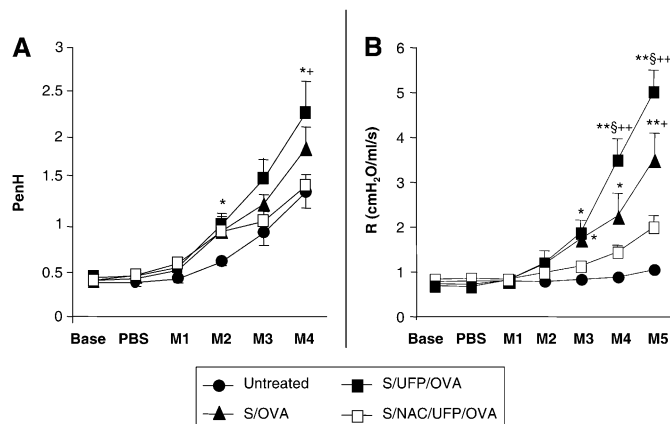


Figure 5. Airway hyperresponsiveness (A) represented by enhanced pause (Penh), measured with a noninvasive body plethysmograph, and (B) by respiratory system resistance (R) measured in intubated mechanically ventilated animals 24 hours after ovalbumin (OVA) challenge. Untreated mice (untreated), OVA-sensitized and -challenged mice (S/OVA), OVA-sensitized mice exposed to elemental carbon ultrafine particles (EC-UFPs) 24 hours before OVA challenge (S/UFP/OVA), and OVA-sensitized mice exposed to EC-UFP 24 hours before OVA challenge treated with N-acetylcysteine (NAC) (S/NAC/UFP/OVA). Data are expressed as mean $\pm$ SEM; (A) $n \geq 9$ per group; (B) $n \geq 4$ per group. * $P < 0.05$, ** $P < 0.01$ versus untreated; + $P < 0.05$, ++ $P < 0.01$ versus S/NAC/UFP/OVA; § $P < 0.05$ versus S/OVA.

major mediators of inflammatory responses, as well as p50/p50 homodimers. Next, to test whether enhanced oxidative stress contributes to S/UFP/OVA and S/OVA increase in NF- κ B activation, lungs of sensitized and challenged mice exposed to EC-UFPs or filtered air and treated with NAC (S/NAC/UFP/OVA, S/NAC/OVA) were analyzed for NF- κ B activation. NAC treatment clearly reduced NF- κ B activation of S/OVA mice and abolished the increase of NF- κ B activation in the lungs of S/UFP/OVA-treated animals (Figures 6A and 6B).

Intracellular Localization of EC-UFP Coincides with High Levels of NF- κ B in the Lung

In sensitized and nonsensitized mice exposed to EC-UFPs, independently from OVA challenge, particles could be detected within the cytoplasm of ciliated cells, pneumocytes type I and type II, alveolar macrophages, and endothelial cells (Figures 7A–7E, S/UFP/OVA), both in the 1-day and 7-day time points. EC-UFP particles were surrounded by a single membrane in all cell types (Figures 7B and 7C, insets). In alveolar macrophages, EC-UFPs were frequently found within phagolysosomes (Figure 7D, inset). No particles could be found in mucous cells. To evaluate if the increase of NF- κ B activation quantified in S/UFP/OVA mice would correspond with the ultrastructural localization of EC-UFPs in the lungs, an immunohistochemical analysis of NF- κ B subunits p50/p65 and p50/p50 was performed on lung sections of S/UFP/OVA mice 7 days after allergen challenge. Nuclear and cytoplasmic staining was apparent for both antibodies, consistent with the activation of NF- κ B pathway. Bronchial and bronchiolar epithelial cells stained positively with both antibodies, excluding the mucous cells (Figures 8A, 8B, and 8G, arrows). The extent of staining of the alveolar epithelium varied. Type I cells stained with p65 only (Figure 8C), but type II cells stained positively with both antibodies (Figures 8C and 8H, large arrows). Endothelial cells stained strongly with p65, and with p50 the stain was strictly nuclear and less consistent compared with p65 (Figures 8A, 8B,

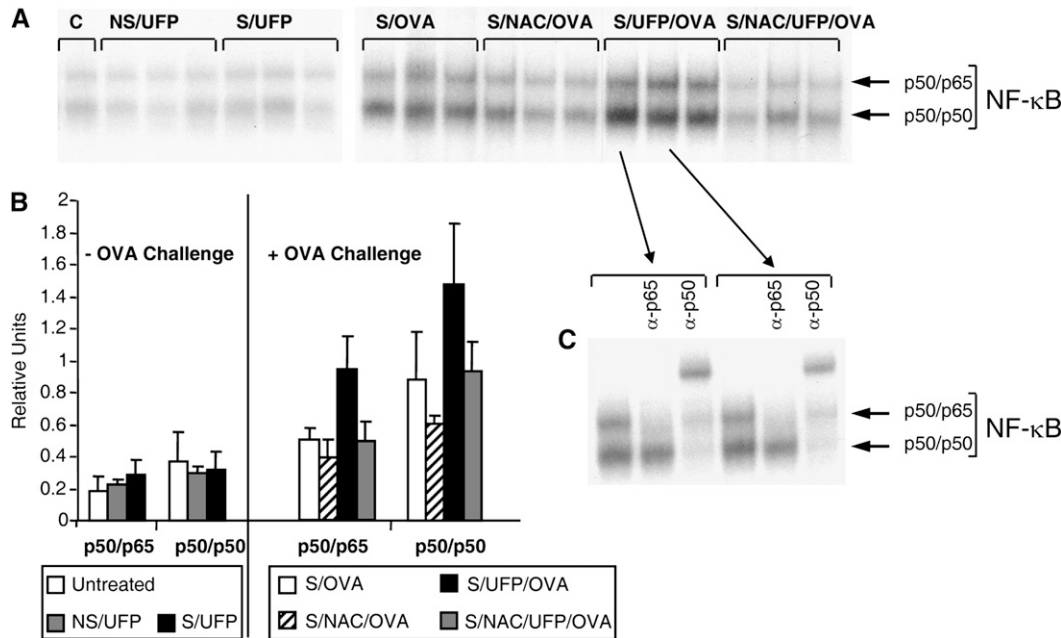


Figure 6. Electrophoretic mobility shift assay (EMSA) showing nuclear factor- κ B activation in mouse lung whole extracts 7 days after elemental carbon ultrafine particle (EC-UFP) exposure. (A) One representative untreated lung and three samples per group for nonsensitized and sensitized mice exposed to EC-UFP (NS/UFP and S/UFP, respectively); sensitized and challenged mice treated or not with N-acetylcysteine (NAC) (S/OVA, S/NAC/OVA) and sensitized and challenged mice exposed to EC-UFP and treated or not with NAC (S/UFP/OVA, S/NAC/UFP/OVA). (B) Densitometric analysis using Aida Image Analyzer. Intensity of bands is expressed in relative units. *Left*, p50/p65 and p50/p50 subunit detection in S/OVA, S/NAC/OVA, S/UFP/OVA, and S/NAC/UFP/OVA. Data are expressed as mean $\pm$ SD; $n = 3$ per group. (C) Supershift EMSA for two representative samples of S/UFP/OVA using antibodies against the two nuclear factor- κ B subunits α -p65 and α -p50.

tion in untreated control mice, NS/UFP and S/UFP. *Right*, p50/p65 and p50/p50 subunit detection in S/OVA, S/NAC/OVA, S/UFP/OVA, and S/NAC/UFP/OVA. Data are expressed as mean $\pm$ SD; $n = 3$ per group. (C) Supershift EMSA for two representative samples of S/UFP/OVA using antibodies against the two nuclear factor- κ B subunits α -p65 and α -p50.

8G, and 8H, *small arrows*). Inflammatory and antigen-presenting cells revealed the strongest staining (Figures 8A–8C and 8G–8H, *arrowheads*, and Figure 8B, *inset*). The immunolocalization of NF- κ B activation coincided with the ultrastructural localization of EC-UFPs. To evaluate if the staining was dependent from EC-UFP inhalation, we performed an immunohistochemical analysis of the NF- κ B subunits p50/p65 and p50/p50 on the lungs of S/OVA mice at the 7-day time point. Bronchial and bronchiolar epithelial cells stained positively with both antibodies (Figures 8D, 8E, 8I, and 8L, *large arrows*). Alveolar epithelial cells and endothelial cells showed no or scarce stain (Figures 8E and 8L). In addition, inflammatory cells stained less consistently compared with S/UFP/OVA (Figures 8D, 8E, 9I, and 8L, *arrowheads*). No staining was detected in

tissue where no antibody or control IgG was used in the primary layer (IgG control, Figure 8F).

DISCUSSION

Increasing clinical, epidemiological, and experimental evidence indicates that airborne PM, especially if it is fine fraction, may contribute to adverse respiratory syndromes, including pulmonary allergic disorders (5, 17, 18, 30–32). Recent experimental evidence focused on the important role of the ultrafine fraction of PM in allergic lung inflammation (17, 18, 33). From these studies the message was clear. Carbonaceous UFPs *per se*, which resemble the core of diesel exhaust, possess an adjuvant activity. The mechanisms responsible for these effects are still

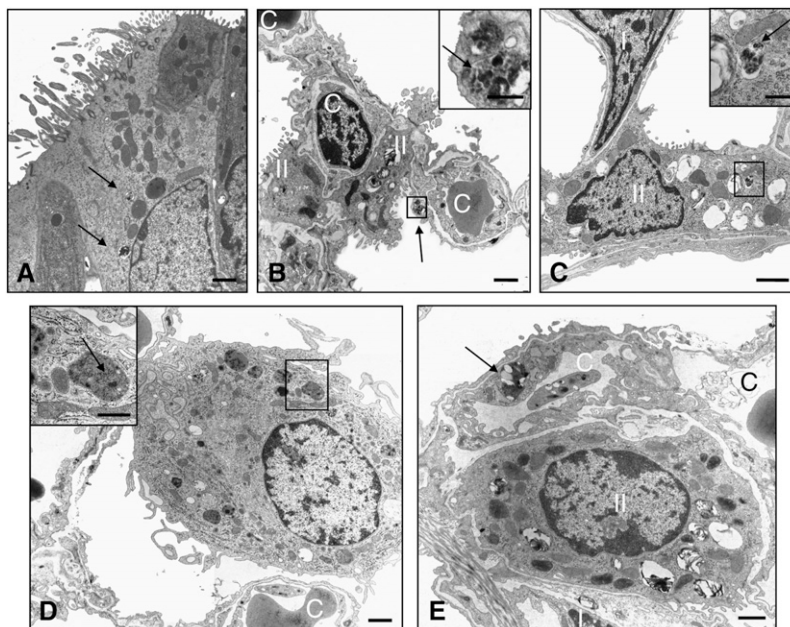


Figure 7. Transmission electron microscopy electron micrographs demonstrating ultrastructural localization of elemental carbon ultrafine particles (EC-UFPs) in lung tissue of sensitized and ovalbumin (OVA)-challenged mice (S/UFP/OVA) 7 days after EC-UFP exposure. EC-UFPs were found in (A) ciliated, (B) type I, and (C) type II epithelium, (D) in alveolar macrophages, and (E) in endothelium. The particles were surrounded by a membrane (*arrows*, *inset* in B and C). In alveolar macrophages EC-UFPs were present also within phagolysosomes (D, *arrow* in *inset*). Scale bar, 1 μ m for A, B, C, D, and E; insets: 250 nm for B and 500 nm for C and D.

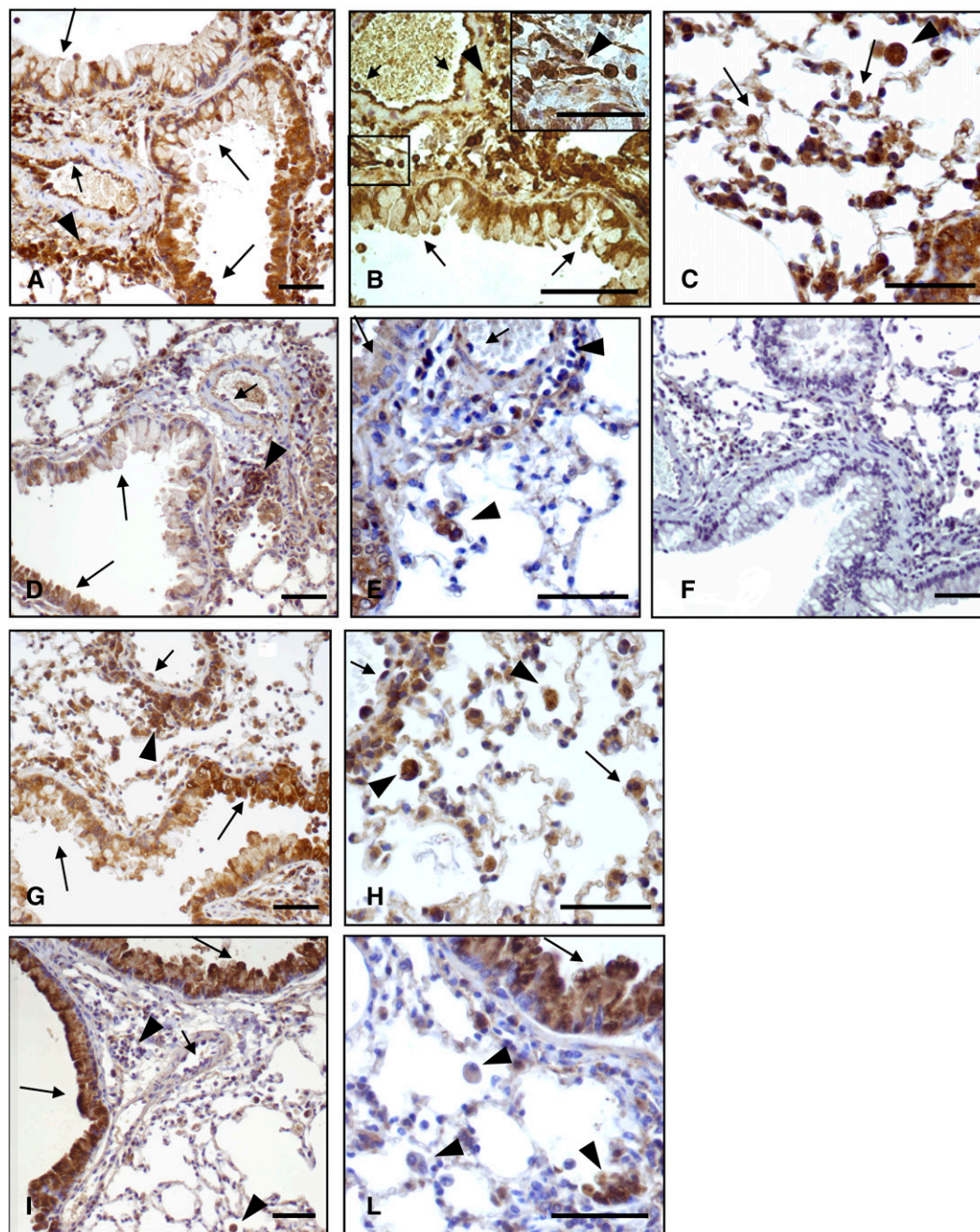


Figure 8. Immunohistochemical analysis was performed in formalin-fixed, paraffin-embedded mouse lungs from sensitized and ovalbumin (OVA)-challenged mice exposed for 24 hours to elemental carbon ultrafine particles (EC-UFPs) (S/UFP/OVA) or to filtered air (S/OVA) and retrieved 7 days after OVA challenge ($n = 2$). Staining of p50/p65 (A–C) in S/UFP/OVA and (D, E) in S/OVA, and of p50/p50 (G, H) in S/UFP/OVA and (I, L) in S/OVA. (Figures 8J and 8K are not shown.) (A, B, D, E, G, I, L, large arrows) Ciliated epithelium, (C and H, large arrows) type II cells, (A, B, D, E, G, H, small arrows) endothelium, (A–E, G–L, arrowheads) inflammatory, and (B, inset, arrowhead) antigen-presenting cells are shown. Both antibodies showed nuclear and cytoplasmic staining. (F) Control IgG showed no stain. Scale bar: 50 μ m for A–L and 25 μ m for B, inset.

a matter of investigation. In this study we used NAC, a thiol compound that acts directly as a free radical scavenger (34) and as a precursor of reduced glutathione (35), to assess the role of oxidative stress in EC-UFP-induced increase of allergic inflammation of the lung. We chose NAC because it is relatively innocuous, it has been widely used both *in vitro* and *in vivo*, and it has a short half-life (36). The antioxidant capabilities of NAC have been demonstrated in an alveolar macrophage cell line (29) as well as *in vivo*. In particular, NAC was able to suppress neutrophilic lung inflammation and NF- κ B activation after endotoxin treatment (27), as well as to prevent lung inflammation after short-term inhalation exposure to concentrated ambient particles (26) or to UFP instillation (12). In addition, NAC has demonstrated its efficiency in inhibiting the adjuvant effect of inhaled fine particulates on allergen sensitization only by daily applications of the compound during the whole sensitization period (29) and failed by using lesser amounts

(37). NAC is in fact known for its short half-life. This property of the compound was particularly important in our experimental setting, because we wanted to restrict the antioxidant capabilities of NAC only to the duration of EC-UFP exposures and avoid unnecessary treatment with the compound. Therefore, we treated the mice twice, shortly before and close to mid-EC-UFP (or filtered air) exposure, with an adequate amount of NAC below its concentration of toxicity of 400 mg/kg body weight. Our results show that in allergen-sensitized and -challenged animals NAC significantly abolishes EC-UFP-induced augmentation of allergen-induced lung inflammatory infiltrate, BAL fluid Th2 cytokine production, and airway hyperresponsiveness. In addition, NAC had a modulatory effect on the allergic inflammation of the lung in the animals exposed to filtered air (S/OVA), which lacked the additional oxidative stress caused by EC-UFP exposure. This was expected, because it is in line with previous studies showing that oxidative stress is

involved in allergic inflammation and asthma (19, 20) and that oral administrations of NAC for 7 consecutive days before allergen challenge have protective effects on allergic airway inflammation (38). We used lower concentrations that nevertheless had an effect on lung allergic inflammation in S/OVA animals.

To confirm that the inhibitory effect of NAC was not due to a mechanism distinct from oxidation-reduction, we repeated the experiment using another antioxidant, buccillamine (BUC, two intraperitoneal injections before and close to mid-EC-UFP [or filtered air] exposure, 20 mg/kg body weight in PBS, 200 μ L [29, 39]). BUC similarly to NAC reduced EC-UFP-dependent increase of inflammatory alterations and mucous cell hyperplasia (Figures E2 and E3).

As a marker for oxidative stress, we chose to measure 8-isoprostane in lung tissue (40). Isoprostanes are a family of eicosanoids of nonenzymatic origin, produced by the random oxidation of tissue phospholipids by oxygen radicals. Elevated levels of isoprostanes have been found in serum, plasma, and urine of heavy smokers (41, 42) and in exhaled condensate of asthmatic patients (43). Our results show that EC-UFP inhalation in nonsensitized animals causes an increase in lung isoprostane concentration 1 day after the termination of the inhalation, lasting until the 7-day time point. Interestingly, at the 1-day time point the increase in isoprostanes concentration is bigger in sensitized animals, suggesting that sensitized mice have an increased susceptibility to EC-UFPs, but only shortly after the exposure. Isoprostane concentration in lung homogenates increased further in sensitized and challenged (S/OVA) animals, reaching the maximum in sensitized and challenged animals exposed to EC-UFPs (S/UFP/OVA). The increased lipid peroxidation in S/OVA mice confirmed previous measurements performed in patients with asthma (43) and asthmatic mice (20). NAC treatment was very effective in reducing lung isoprostane levels in all experimental groups. This effect was most prominent in mice that had been exposed to EC-UFPs. These data confirmed the important involvement of oxidative stress in EC-UFP-induced injury, both in normal and in allergic animals.

NF- κ B is an important intracellular signaling pathway for both innate and acquired immunity and is involved in proinflammatory gene transcription (44). How oxidative stress is involved in NF- κ B activation and at which level NAC acts as inhibitor of NF- κ B are still matters of debate (45–47). Oxidative stress can directly or indirectly activate the transcription factor NF- κ B by phosphorylation of I κ B and induce translocation of NF- κ B into the nucleus, a process that is followed by transcriptional activation of many inflammatory cytokine and chemokine genes. To have an insight into the mechanisms underlying the oxidative stress-induced adjuvant activity of EC-UFPs, we evaluated NF- κ B activation in lung homogenates. Preliminary data showed no additional activation of NF- κ B in allergic mice exposed to EC-UFPs (S/UFP/OVA) versus filtered air (S/OVA) evaluated 1 day after OVA challenge. Strong differences in NF- κ B activation between S/UFP/OVA and S/OVA were detected 7 days after OVA challenge, suggesting that the inflammatory reaction that we observed 7 days after OVA challenge was sustained by NF- κ B activation. A moderate NF- κ B activation was found in NS/UFP mice immediately after EC-UFP exposure (Figure E1), whereas no significant activation of NF- κ B was observed in nonchallenged mice (NS/UFP or S/UFP) 7 days after EC-UFP exposure. This observation is in line with *in vitro* data showing transitional early time points of NF- κ B activation in rodent cell lines exposed to fine particulates (22, 48). Our observation of the lack of NF- κ B activation in NS/UFP and S/UFP in the latest time point corresponded well to the lack of inflammatory infiltrate but was not in line with the increase in lipid peroxidation observed in both groups. A

potential explanation for this discrepancy may be found in the delicate balance between oxidative and antioxidative mechanisms. Oxidative stress also activates transcriptional factors, such as Nrf-2, which can mediate the transcriptional regulation of phase II antioxidant genes (49). It thus may be speculated that in nonchallenged mice the antioxidant protective genes were enough to counterbalance the increase in oxidative stress induced by EC-UFP exposure at early time points. In contrast, allergen challenge and EC-UFP exposure in sensitized mice seem to induce an oxidative stress of a higher magnitude that cannot be counterbalanced by endogenous antioxidative mechanisms and results in a loss of the redox balance, with consequent NF- κ B activation and augmentation of the inflammation.

Whether reactive oxygen species are directly involved in the triggering of NF- κ B by EC-UFPs has yet to be elucidated.

Our evaluation of EC-UFP localization by electron microscopy revealed presence of EC-UFPs and their agglomerates in bronchiolar and alveolar epithelial cells, macrophages, and endothelial cells at 1 and 7 days after the termination of EC-UFP exposure, confirming the relatively fast process of EC-UFP translocation to the vasculature (7). The cells, which at the evaluation by transmission electron microscopy contained EC-UFPs, coincided with the cells that displayed NF- κ B activation in immunohistochemistry. From the difference in staining between S/UFP/OVA and S/OVA lungs we can assume that EC-UFPs induce additional NF- κ B activation of alveolar macrophages, alveolar epithelial cells, and endothelial cells, which may be in part responsible for the adjuvant activity of the particles.

In this study we demonstrated that inhalation of relatively inert EC-UFPs caused oxidative stress in the lung. Sensitized and challenged mice also displayed NF- κ B activation, further increased by EC-UFP exposure, localized in inflammatory, epithelial, and endothelial cells. The potent effect of NAC treatment confirmed that EC-UFP inhalation has potentiating effects in causing oxidative stress in lung allergic inflammation. For S/UFP/OVA we can hypothesize that the resulting inflammatory response can increase allergen penetration during the subsequent challenge phase.

Asthma is a disease characterized by lowered antioxidant defenses and therefore increased susceptibility to oxidative stress. Metabolic and genetic studies of allergic patients sustain this observation (50, 51). For people with asthma, particle exposure could then exacerbate the already existing difficulty to counteract reactive oxygen species. Our data support the concept that allergic individuals are more susceptible to the adverse health effects of EC-UFPs.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Role of Oxidative Stress on Ultrafine Particle-Induced Exacerbation of Allergic Lung Inflammation

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Online data supplement

Allergen sensitization/challenge protocol

In order to evaluate the adjuvant effects of EC-UFP inhalation, a protocol of mild allergic inflammation of the lung was employed. Mice were sensitized by repetitive intraperitoneal injections of 1 μ g OVA (Sigma, grade VI)/Alum (Pierce, 2.5 mg) in phosphate buffered saline (PBS) on days 0, 7, 14, 28, 42, 56. Blood samples were taken before and after sensitization. OVA-specific IgE and IgG1 were measured in plasma samples by ELISA as described previously (1). OVA/alum sensitized mice (day 63), compared to non-sensitized controls, were characterized by high titers of OVA-specific IgE (31.7 ± 3.3 vs 0.1 ± 0.01 μ g/ml) and OVA-specific IgG1 (1178.4 ± 211.2 vs <0.1 μ g/ml). On day 70 the mice were aerosol-challenged for 20 min with 1%-LPS-depleted (1) OVA in PBS or with PBS alone delivered by a Pari-Boy nebulizer (Pari, Starnberg, Germany).

EC-UFP production and characterization

EC-UFP were generated by electric spark discharge (model GFG1000, Palas, Germany) providing agglomerated carbon particles as previously described (1). EC-UFP average size distributions were characterized by a count median diameter of 53.2 nm with a geometric standard deviation of 1.53 and an average mass median diameter of 80.4 nm. Particle mass distribution was calculated from scanning mobility particle sizer (SMPS) number distribution data (Model 3071, TSI Inc., Shoreview, MN, USA) using particle effective density (2, 3). Spark generated EC-UFP are considered to be structurally similar and form aggregates similarly to the primary particles of modern diesel emissions (4, 5). When investigating the chemical nature of the thermally desorbed gases by evolved gas analysis with mass spectrometry and Fourier transform analysis, less than 5% of the desorbed mass was identified as organic (6). This is in line with Frampton et al. who used a time-of-flight mass spectrometer and confirmed that at least 96% of the mass of UFP (generated with the same improved spark generator from Palas) consisted of elemental carbon (7). Spark generated EC-

UFP are considered then to be relatively inert particles compared to other particles such as DEP and concentrated atmospheric particles (CAPS). We chose them for the inhalation exposure because of their defined chemical composition and stable physical characteristics during the 24 exposure experiment.

Animals were housed in 330 liter exposure chambers with a horizontal displacement flow of 100 lpm (18.2 exchanges of the chamber air/hour, 5 mm s⁻¹ average displacement velocity).

Bronchoalveolar lavage (BAL) analysis

Airways were lavaged five times with 0.8 ml PBS. Aliquots of cell-free BAL fluid were assayed in duplicate for total protein (Coomassie Protein Assay, Pierce Chemical Co.) and for IL-5, IL-13, IL-10 and IFN- γ by two site ELISAs using antibodies from BD Biosciences (Heidelberg, Germany) and BioSource (IL-13, Camarillo, CA, USA) as suggested by the manufacturer. Viability and yield of BAL cells was quantified via trypan blue (Serva, Germany) exclusion in a hemocytometer. Differential BAL cell count (400 cells/sample) was performed on cytopins (600 rpm for 10 min) fixed and stained with Diff-Quick (Dade Behring, Marburg, Germany).

Histological and immunohistochemical evaluation

After BAL the left lobes of 2 lungs/time point/group were removed and fixed in 10% buffered formalin. After paraffin embedding, 5 μ m sections were cut. For histological analysis, the sections were stained with hematoxylin-eosin (H&E) and periodic acid Schiff (PAS). Mucus hypersecretion and inflammatory cell infiltration were evaluated in a blinded fashion. For immunohistochemistry, after deparaffinization the sections were placed in 0.1 M citric acid, pH 6.0, and heated in a microwave at low power for 15 min; immunostaining was performed using an ABC-based method (Vector Laboratories, Burlingame, CA) as previously described (8). As primary antibodies, α -NF- κ B p50 (sc-114) and α -NF- κ B p65 (sc-372) were

used (Santa Cruz Biothechnology, Inc., Santa Cruz, CA, USA). Negative controls included use of buffer alone or respective dilutions of non-specific purified rabbit immunoglobulin G (IgG) in the primary layer (Vector Laboratories).

Intracellular localization of EC-UFP by transmission electron microscopy

Lungs were fixed by intratracheal perfusion with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4); the peripheral lung was minced and immersed in the same fixative for 4-7 days at 4°C, post fixed in 1% osmiumtetroxide in cacodylate buffer for 1 h at 4°C and embedded in EMbed 812 (Science Services, München, Germany). After tissue evaluation on semithin sections, silver coloured ultrathin sections were mounted on formvar-coated 75-mesh nickel grids, double-stained with uranyl acetate and lead citrate and observed in a transmission electron microscope (JEOL 1210, Tokyo, Japan) at 80 kV.

Lung function tests

Lung function tests were performed 24 h after allergen challenge with two different techniques: in unrestrained animals using whole body plethysmography (Buxco® Electronics, Sharon, Connecticut, Fig. 5 a) and in intubated, mechanically ventilated animals (Fig. 5b). For whole body plethysmography, the 'enhanced pause' (PenH) determined before and after methacholine exposure was applied as an index of airway hyperresponsiveness (AHR), as previously described (1, 9). The doses of methacholine aerosol (M1-M4) delivered to the mice were the following: 10 mg/ml methacholine nebulized for 1, 2 and 4 minutes (M1-M3), followed by 40 mg/ml methacholine for 2 minutes (M4). A data recording interval of 3 min was introduced after each methacholine level. The mean of the PenH values determined in the 2nd and 3rd min was used for quantifying AHR.

For the measurement of pulmonary mechanics, anesthesia was induced by inhalation of isoflurane (5%) in O₂ for 1 min in a whole body box and maintained by an intraperitoneal

injection of a mixture of medetomidine (0.15 mg/kg), midazolam (2.0 mg/kg), and fentanyl (0.005 mg/kg). The trachea was cannulated (28 G i.v. catheter, Sims Portex Ltd, Hythe, England) and the animals were ventilated with an air/1% isoflurane mixture at rates of 120 - 130 breaths/minute as previously described (10, 11). The tail vein was cannulated for the delivery of methacholine. Muscle relaxation was achieved by an intraperitoneal injection of pancuronium (1 mg/kg). Measurements of lung volumes and baseline pulmonary mechanics were performed as previously described (10). Respiratory system resistance (R) was derived from recordings of flow and airway opening pressure (Pao) during a standardized breath (130 breaths/minute, tidal volume at 40% of inspiratory capacity). R was calculated from the change in Pao and flow at isovolumetric points (mid-inspiratory and mid-expiratory volume) and corrected for the resistance of the cannula (10). To obtain dose-response curves to iv methacholine, mice were given a slow inflation to a Pao of 25 cmH₂O. One minute later, PBS was administered intravenously (1µl/g body weight) and R was measured repeatedly for the next 5 minutes until R peaked and began to decline. The mice were then given another inflation to 25 cmH₂O. The procedure was repeated using increasing doses of methacholine dissolved in PBS delivered in half-log intervals (M1-M5) from 0.04-4 mg/ml at a dose of 1 µl/g body weight. The three highest values of R obtained following each dose were averaged to obtain final R values for each dose.

Measurement of 8-isoprostane

The concentration of 8-isoprostane was determined in all lung homogenates after BAL. Homogenates were prepared by disrupting lung tissue in Hepes, pH 7.4, with lysing matrix E (FastPrep FP120 cell disrupter; MP Biomedicals Germany GmbH, Eschwege, Germany). After centrifugation (3200 g at 4°C for 10 min), aliquots of the obtained supernatants were taken for determination of protein and 8-isoprostane. For analysis of 8-isoprostane, supernatants were deproteinized by adding 8-fold volume of 90 % methanol containing 0.5

mM EDTA and 1 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, pH 7.4 according to Beck-Speier et al (12); 8-isoprostane concentration was determined by specific enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI, USA) as suggested by the manufacturer. Protein was measured at 595 nm using 5 µl of homogenate and 200 µl of 1:5 diluted Biorad solution (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

Electrophoretic mobility shift assay (EMSA) for NF-κB activation

To determine NF-κB activation, whole lung tissue was homogenized in high salt buffer [20 mM HEPES pH 7.9, 350 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM MgCl₂, 10% Glycerol, 1% Nonident P-40 (Fluka, Chemie AG, Basel, Switzerland), 10 mM NaF, 8 mM β-glycerophosphate (Calbiochem Merk, Darmstadt, Germany), 1 mM DTT and protease inhibitor cocktail (Roche Diagnostics, GmbH, Mannheim, Germany)]. After 2 hours of incubation at 4°C, the lysate was centrifuged for 15 min at 13.000 r.p.m. in a microfuge. EMSA was performed as previously described (13). A densitometrical analysis (Aida Image Analyzer, v3.51) served to quantify NF-κB activity relatively to the positive control Oct 1. The background intensity was subtracted from the calculated intensity of each band. The composition of NF-κB complexes was analysed by supershift assay using α-p50 (sc-114) and α-p65 (sc-372) antibodies (Santa Cruz Biotechnology).

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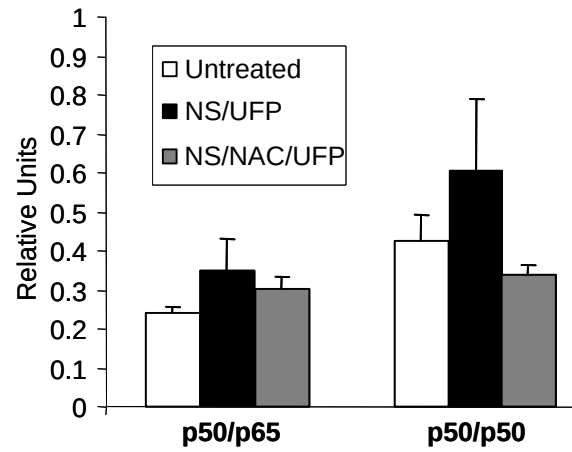


Fig. E1: Densitometrical analysis (Aida Image Analyzer) of EMSA showing NF-kB activation in lung whole extracts of non sensitized mice immediately after EC-UFP exposure (NS/UFP, time 0). Intensity of bands is expressed in relative units. p50/p65 and p50/p50 subunit detection in untreated controls, NS/UFP and NS/NAC/UFP. Data are expressed as mean $\pm$ SD; n=3/group.

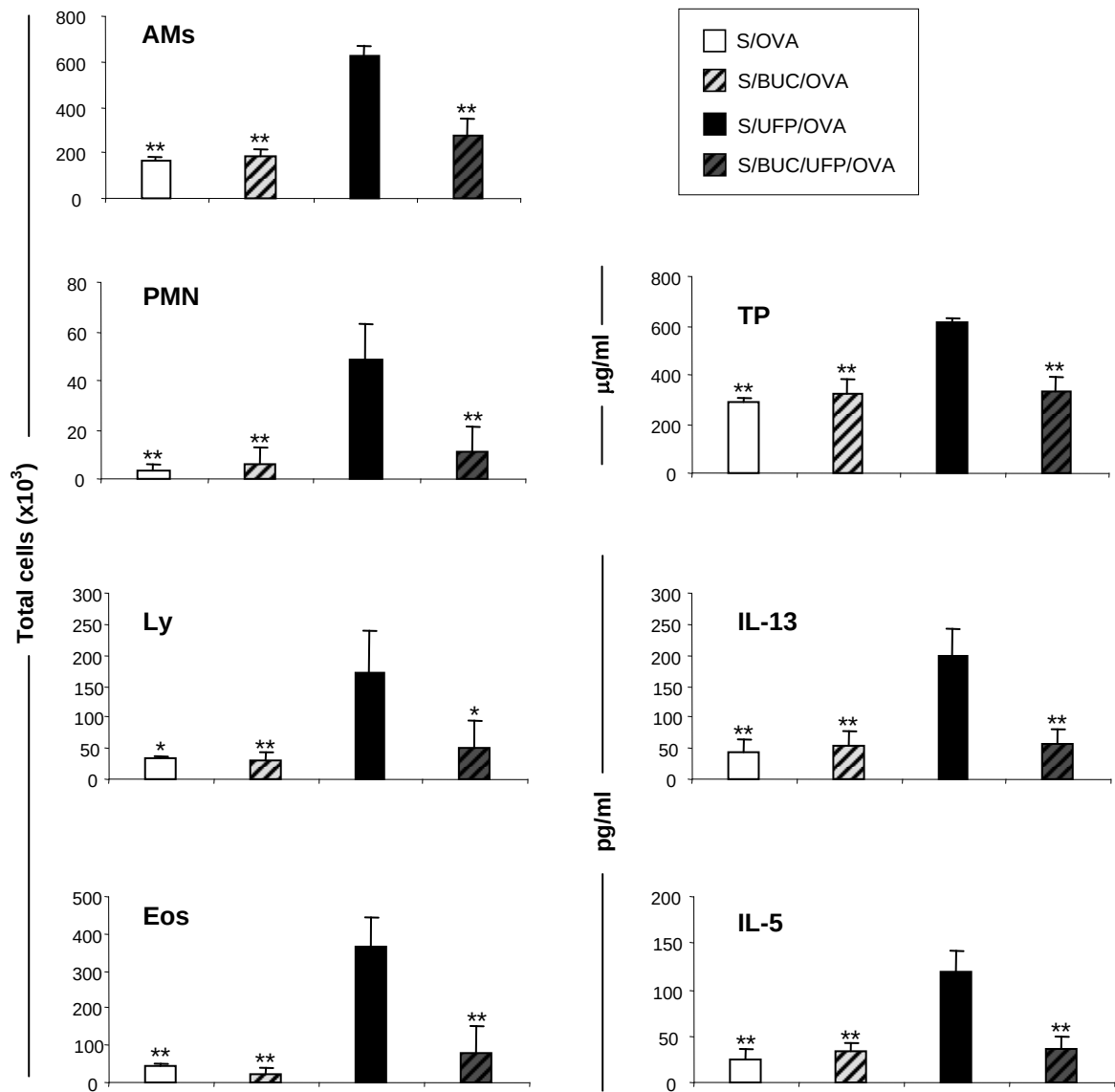


Fig. E2: Effect of bucillamine (BUC) on BAL Cells (left) and BAL fluid total protein (TP) and cytokines (right) in OVA sensitized mice 7 days after OVA challenge. BUC was administered with two intraperitoneal injections (20 mg/kg body weight in PBS, 200 μ l) prior to and close to mid EC-UFP (or filtered air) exposure. The following groups are depicted in the figure: sensitized and challenged mice exposed for 24h to filtered air (S/OVA), treated with BUC (S/BUC/OVA); exposed to EC-UFP for 24h prior to OVA challenge (S/UFP/OVA), and treated with BUC (S/BUC/UFP/OVA). Data are expressed as mean $\pm$ SD; n=3-5/group. *p<0.05, **p<0.01 vs S/UFP/OVA.

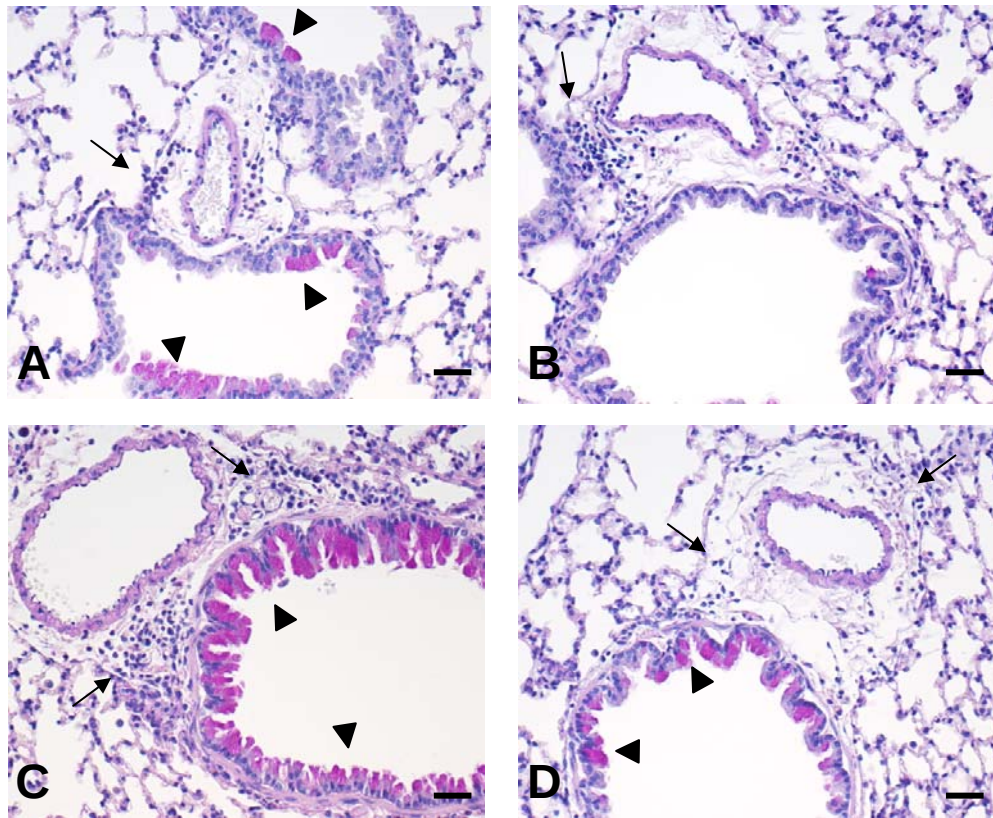


Fig. E3: Effect of bucillamine (BUC) on lung histology (PAS staining) 7 days after OVA challenge in representative samples: A) sensitized and challenged mice exposed to filtered air (S/OVA); B) sensitized and challenged mice exposed to filtered air and treated with BUC (S/BUC/OVA); C) sensitized mice exposed to EC-UFP for 24h prior to OVA challenge (S/UFP/OVA); D) sensitized mice exposed to EC-UFP for 24h prior to OVA challenge and treated with BUC (S/BUC/UFP/OVA). Arrows, inflammatory infiltrate; arrowheads, mucus hypersecretion; scale bar, 32 μ m.