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Exposure to welding fumes and lower airway infection with *Streptococcus pneumoniae*

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Abstract

Background—Welders are at increased risk of pneumococcal pneumonia. The mechanism for this association is not known. The capacity of pneumococci to adhere to and infect lower airway cells is mediated by host-expressed platelet-activating factor receptor (PAFR).

Objective—We sought to assess the effect of mild steel welding fumes (MS-WF) on PAFR-dependent pneumococcal adhesion and infection to human airway cells *in vitro* and on pneumococcal airway infection in a mouse model.

Methods—The oxidative potential of MS-WF was assessed by their capacity to reduce antioxidants *in vitro*. Pneumococcal adhesion and infection of A549, BEAS-2B, and primary human bronchial airway cells were assessed by means of quantitative bacterial culture and expressed as colony-forming units (CFU). After intranasal instillation of MS-WF, mice were infected with *Streptococcus pneumoniae*, and bronchoalveolar lavage fluid (BALF) and lung CFU values were determined. PAFR protein levels were assessed by using immunofluorescence and immunohistochemistry, and PAFR mRNA expression was assessed by using quantitative PCR. PAFR was blocked by CV-3988, and oxidative stress was attenuated by N-acetylcysteine. Results: MS-WF exhibited high oxidative potential. In A549 and BEAS-2B cells MS-WF increased

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pneumococcal adhesion and infection and PAFR protein expression. Both CV-3988 and N-acetylcysteine reduced MS-WF–stimulated pneumococcal adhesion and infection of airway cells. MS-WF increased mouse lung PAFR mRNA expression and increased BALF and lung pneumococcal CFU values. In MS-WF–exposed mice CV-3988 reduced BALF CFU values.

Conclusions—Hypersusceptibility of welders to pneumococcal pneumonia is in part mediated by the capacity of welding fumes to increase PAFR-dependent pneumococcal adhesion and infection of lower airway cells.

Keywords

Occupational disease; welding fumes; platelet-activating factor receptor; Streptococcus pneumoniae; pneumonia; bacterial adhesion and infection

Occupational data from England and Wales for 1970 to 1972 report there were 66 deaths among welders compared with 42 expected deaths.[1] Similar data for 1990 to 2000 suggest that excess deaths among welders are due to pneumonias other than bronchopneumonia, principally lobar pneumonia, and are present in other occupations associated with exposure to metal fumes.[2] Hypersusceptibility to pneumonia appears to be reversible because excess deaths are limited to welders of less than the normal retirement age,[2] and a recent United Kingdom(UK) case-control study found that hospital admissions for community-acquired pneumococcal pneumonia in working-age men were associated with occupational exposure to metal fumes in the past year but not in earlier periods.[3] Increased risk of pneumonia in welders has also been found outside the UK. For example, in a cohort of more than 30,000 Swedish construction workers with exposure to metal fumes, Toren et al[4] reported that mortality from lobar pneumonia was 3.7-fold higher and mortality from pneumococcal pneumonia was 5.8-fold higher relative to their peers. By contrast, deaths from pneumonia in retired metal workers were only marginally increased.[4] Although these findings suggest that inhalation of welding fumes (WF) increases the risk of pneumococcal infection, the high prevalence of other exposures in welders associated with increased risk of pneumococcal disease, such as smoking,[5,6] and the lack of a biologically plausible mechanism result in uncertainties about causality. However, animal studies reporting that WF impair pulmonary clearance of *Listeria monocytogenes*[7–9] suggest that WF have the potential to adversely affect the pulmonary innate immune system.

Adherence of pneumococci to lower airway cells is a first step in the development of airway infection leading to pneumonia.[10] For *Streptococcus pneumoniae* (and other phosphorylcholine-expressing bacteria, such as nontypeable *Haemophilus influenzae*[11] and *Acinetobacter* species[12]), adhesion and infection of lower airway cells is facilitated by an interaction between bacterial phosphorylcholine and the platelet-activating factor receptor (PAFR; 10-alkyl-2-acetyl-glycerophosphocholine PAF) expressed on host cells.[13] Because previous studies report that inhaled toxins, including fossil fuel–derived particulate matter (PM) and cigarette smoke,[14,15] through induction of oxidative stress, upregulate PAFR-dependent adhesion of pneumococci to airway epithelial cells, we hypothesized that hypersusceptibility to pneumonia in welders is mediated through PAFR-dependent pneumococcal adhesion. Therefore in this study we sought to assess the oxidative potential (OP) of mild steel welding fumes (MS-WF), the effect of MS-WF on PAFR-dependent

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pneumococcal adhesion and infection in human lower airway cells *in vitro*, and PAFR-dependent pneumococcal airway infection in a mouse model. We also assessed PAFR in stored lung tissue from a study in which mice were exposed to aerosolized stainless steel welding fumes (StS-WF) and from a study of particles in welders' lungs.[16]

METHODS

WF: Generation and composition

MS-WF were a gift from the Welding Institute (Cambridge, UK). MS-WF were obtained by using a standardized method in accordance with the International Standard 15011-1:2009, as previously described.[17] Briefly, manual metal arc welding electrodes (mild steel E7018 basic type) were run to produce a weld bead inside a fume collection system. MS-WF with a mode particle diameter of 6.8 μm[18] were extracted through the hood on top of the box, collected on a filter paper, removed by brushing, and stored in airtight glass containers. The composition of MS-WF was determined after digestion in nitric/hydrochloric acid in a high-temperature, closed-vessel, microwave-assisted dissolution system. Analysis was done by using inductively coupled plasma–atomic emission spectroscopy. Before use, MS-WF were suspended in PBS.

WF: OP

The OP of MS-WF was determined based on their ability to oxidize antioxidants from a validated *in vitro* respiratory tract lining fluid model containing equimolar (200 μmol/L) and physiologically relevant concentrations of ascorbate, urate, and glutathione.[19] Incubations were performed with particle suspensions at a final concentration of 50 μg/mL for 4 hours at 37°C (pH 7.4) in parallel to particle-free and PM controls (an oxidatively inert carbon black [M120] and an oxidatively active urban PM [NIST1648a]). At the end of this period, particles were removed by means of centrifugation (13,000 rpm at 4°C), and samples were acidified with metaphosphoric acid (final concentration 5%) before determination of the remaining antioxidant concentrations by using reverse-phase HPLC with electrochemical detection (for ascorbate) and the glutathione disulphide-reductase-5, 5′-dithio-bis (2-nitrobenzoic acid) recycling assay (for glutathione).[19] OP was determined based on the percentage loss of ascorbate and glutathione over the 4-hour incubation period relative to a 4-hour particle-free control (reflecting background auto-oxidation rates). Under these conditions, urate losses are not significant.[16,17] The percentage loss of ascorbate and glutathione over the 4-hour incubation was then normalized to the particle concentration used in the respiratory tract lining fluid assay (50 μg/mL) to generate 2 separate measures of OP: glutathione-dependent OP ($OP^{glutathione}$) per microgram and ascorbate-dependent OP ($OP^{ascorbate}$) per microgram. In addition, an aggregate sum of the 2 measures was calculated (OP^{total} per microgram),[20] previous work having shown that ascorbate and glutathione oxidation is sensitive to different panels of oxidants.[16,17]

Pneumococcal adhesion and infection: Human airway cells

A549 cells, a type II pneumocyte cell line (Sigma-Aldrich, Poole, UK),were maintained in Dulbecco modified Eagle medium supplemented with FBS, L-glutamine, and antibiotics (Lonza, Basel, Switzerland). Passage number was less than 20. BEAS-2B, a bronchial

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epithelial cell line, was a gift from Dr Nicolas Mercardo (National Heart and Lung Institute, Imperial College London, London, UK). BEAS-2B cells were maintained in RPMI-1640 medium containing HEPES (Life Technologies, Warrington, UK) supplemented with FBS L-glutamine and antibiotics. Passage number was less than 20.

Cell viability was assessed by using the lactate dehydrogenase (LDH) assay (Sigma-Aldrich), according to the manufacturer's instructions. Cells treated with distilled water (indicating 100% LDH release) were used as a positive control. Primary human bronchial epithelial cells (purchased from Promocell, Heidelberg, Germany; lot no. 4032402) were maintained according to the manufacturer's instructions. Passage number was less than 4. The type 2 *S pneumoniae* encapsulated strain D39 was purchased from the National Collection of Type Cultures (NCTC 7466; Central Public Health Laboratory, London, UK) and grown in liquid culture brain-heart infusion broth (Oxoid, Basingstoke, UK) to the midlogarithmic phase (OD_{600} =0.4–0.6) before use.

Pneumococcal adhesion and infection, and infection alone of airway cells were assessed by using a standard *in vitro* assay.[14,15] Briefly, airway epithelial cells were cultured with MS-WF for 2 hours, washed, and infected with *S pneumoniae* at a multiplicity of infection of 100 for 2 hours to assess the combination of pneumococcal adhesion and infection of cells. Cells were then vigorously washed, detached, and lysed with sterile distilled water. Serial dilutions of the samples were plated on brain-heart infusion agar containing 5% horse blood (Oxoid), and colony-forming units (CFU) per milliliter were assessed. In this assay CFU values after cell lysis reflect both pneumococci attached to the surfaces of airway cells (ie, the adherent fraction) and pneumococci that penetrate into cells (ie, the infective fraction). The adherent fraction was first killed with gentamicin (200 mg/mL) and penicillin G (10 mg/mL), to assess the infective fraction alone. Intracellular pneumococci that were protected from antibiotics were recovered by means of cell lysis with ice-cold sterile water, and CFU values were determined.[15] The functional role of PAFR was assessed by adding a specific PAFR blocker, (RS)-2-methoxy-3-(octadecylcarbamoyloxy)-propyl2-(3-thiazolio) ethylphosphate (CV-3988),[21] at a final concentration of 20 μmol/L. The role of oxidative stress was assessed by adding the thiol antioxidant N-acetylcysteine (NAC; Sigma-Aldrich)[22] at a final concentration of 5 mmol/L at the same time as MS-WF.

Pneumococcal infection: Mouse model

Four- to 6-week-old female CD1 mice (Charles River, Welwyn Garden City, UK) were exposed to MS-WF in 50 μL of PBS (administered either as a single 600-μg dose or as divided doses) through intranasal installation after achievement of isoflurane anesthesia. Twenty-four hours after instillation of MS-WF, animals were intranasally infected with 5×10^6 *S pneumoniae* strain D39 in 50μL of PBS. Animals were killed at 24 hours after pneumococcal infection by using a pentobarbital overdose. Pneumococcal CFU values in bronchoalveolar lavage fluid (BALF), lung tissue (done after BAL), and blood were assessed by plating serial dilutions on brain-heart infusion agar containing 5% horse blood (Oxoid). Mouse experiments were approved by University College London's Biological Services Ethical Committee under UK Home Office Project License PPL70/6510 and performed according to UK national guidelines for animal use and care under UK Home

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Office license in accordance with European Union Directive 2010/63/EU. Animals received 30 μL of 5 mg/kg of the PAFR blocker CV-3988 (Sigma-Aldrich) administered by means of tail vein injection 1 hour before pneumococcal infection to assess the effect of blocking PAFR.

PAFR expression: Human airway cells

Expression of PAFR protein by airway cells *in vitro* was quantified by means of florescence microscopy. Briefly, 4×10^5 cells were grown on cover-slips in 24-well plates and cultured with MS-WF. Cells were fixed in ice-cold 4% paraformaldehyde for 10 minutes at room temperature and washed with PBS with 10% FCS (wash buffer). Cells were exposed overnight at 4°C to either a mouse anti-human PAFR IgG_{2a} antibody (1:100, CAY160600; Cayman Chemicals, Ann Arbor, Mich) or a mouse IgG isotype control (Bio-Legend, San Diego, Calif). Cells were washed with wash buffer and an Alexa Fluor 488–conjugated goat anti-mouse antibody applied (1:1000; Invitrogen, Grand Island, NY) at room temperature for 30 minutes under aluminum foil. Cells were washed with wash buffer and 4′, 6-Diamidino-2-Phenylindole, Dilactate (1:1000, Invitrogen) applied at room temperature for 15 minutes under aluminum foil. Finally, cells were washed with wash buffer, and the coverslips were mounted on glass slides and sealed. Slides were left to air dry under foil for 4 hours and stored overnight at 4°C for analysis. Images were taken with an epifluorescence microscope and analyzed by using ImageJ software (National Institutes of Health, Bethesda, Md). The isotype control confirmed that interactions between the anti-human PAFR antibody and the secondary antibody detected by using immunofluorescence microscopy were specific. Images were obtained from 3 randomly selected areas of each slide and analyzed blind to exposure status. By using the software, a florescence intensity threshold was set to discount background nonspecific florescence. The area of specific florescence was then measured for each image, with 1 to 3 images analyzed in each experiment and expressed as square micrometers.

PAFR mRNA expression: Mouse model

Expression of mouse lung PAFR mRNA was assessed by means of quantitative PCR. Briefly, lungs were removed and stored in RNAlater (Qiagen, Manchester, UK) at −80°C. RNA was extracted with the RNeasy Kit (Qiagen). First-strand cDNA synthesis was carried out with SuperScript VILO MasterMix (Life Technologies). Real-time PCR was carried out with TaqMan Gene Expression MasterMix (Life Technologies). mRNA analysis was carried out according to the manufacturer's instructions by using relative quantification involving normalization to a reference gene. Primer/ probe sets used were as follows: mouse reference gene β_2-microglobulin, Mm00437762_m1; mouse PAFR, Mm02621061_m1 (Life Technologies). All primer/probe sets spanned exon-exon boundaries to control for genomic DNA contamination.

PAFR: Stored samples

The effect of aerosolized WF on mouse lung PAFR mRNA expression was assessed by using tissue samples from mice exposed to aerosolized StS-WF. Mouse lung tissue was obtained from 6-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, Me) exposed

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by means of whole-body inhalation to 40 mg/m^3 StS-WF for 3 hours per day for up to 10 days. Lung PAFR mRNA expression was compared between air-exposed and StS-WF–exposed controls at both 4 hours and 28 days after the last dose. The design and construction of the aerosol generator and the characterization of StS-WF have been previously described.[23] Full details are provided in the Methods section in this article's Online Repository at www.jacionline.org.

The distribution of airway PAFR in a nonsmoking welder and a nonsmoking non–WF-exposed control subject was assessed by immunostaining samples from a study in which normal tissue was obtained at the time of a clinical biopsy for suspected cancer.[16] Full details are provided in the Methods section in this article's Online Repository. Previous sampling and present analysis of human lung tissue was approved by an institutional review board for human studies.[16]

Statistical analysis

Statistical analysis was done with GraphPad Prism software (version 5.03; GraphPad Software, La Jolla, Calif). Data were obtained from at least 3 separate experiments performed at different times, with each data point representing the mean of at least 3 replicates, unless otherwise stated. Data from *in vitro* airway epithelial experiments were analyzed by using either the *t* test or 1-wayANOVAand the Tukey multiple comparison test and are summarized as means (SEMs). Data from animal experiments are summarized by medians and analyzed either by using the Mann-Whitney test or Kruskal-Wallis test and the Dunn multiple comparison test. A *P* value of less than .05 was considered significant.

RESULTS

WF: OP

MS-WF contained iron, manganese, titanium, aluminum, and zinc (Table I). The OP of MW-WF for ascorbate and glutathione was increased compared with that of carbon black, and the total OP of MS-WF was increased compared with that of urban PM (Fig 1).

Pneumococcal adhesion and infection: Human airway cells

We first performed dose-response experiments with A549 and BEAS-2B cells to determine the optimal concentration of MS-WF that stimulated adhesion without causing cytotoxicity. MS-WF at concentrations between 200 and 400 μg/mL for 2 hours increased pneumococcal adhesion and infection of both airway cell lines (Fig 2) without causing cytotoxicity, as assessed based on LDH release (see Fig E1 in this article's Online Repository at www.jacionline.org). A lower concentration of MS-WF (10 μg/mL) stimulated pneumococcal adhesion and infection, but this required extending culture duration to 24 hours (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus we chose to expose cells to MS-WF for 2 hours at 275 μg/mL (145 μg/cm^2) for A549 cells and 200 μg/mL (105 μg/cm^2) for BEAS-2B cells. By adding antibiotics to kill the fraction of pneumococci adherent to cell surfaces, it was determined that MS-WF for 2 hours also increased the infective pneumococcal growth (data not shown).

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Pneumococcal infection: Mouse model

Intranasal instillation of a single 600-μg dose of MS-WF in mice 24 hours before pneumococcal infection resulted in an increase in BALF and lung pneumococcal CFU values at 24 hours after pneumococcal infection (Fig 4). BALF and lung pneumococcal CFU values were also increased when MS-WF was administered as 6 separate 100-μg doses once a day for 6 days (total, 600 μg), followed by infection 24 hours after the last dose (see Fig E3 in this article's Online Repository at www.jacionline.org). In this model pneumococci were not isolated from the blood.

PAFR-dependent adhesion and infection: Human airway cells

A549 and BEAS-2B cell culture with MS-WF for 2 hours increased PAFR protein expression (Fig 5). The addition of CV-3988 to MS-WF–exposed cells reduced pneumococcal adhesion and infection of A549 and BEAS-2B cells and of human primary bronchial epithelial cells (Fig 6). Adding NAC at the same time as MS-WF attenuated pneumococcal adhesion and infection of A549 and BEAS-2B cells (Fig 7).

PAFR-dependent infection: Mouse model

A single intranasal dose of 600 μg of MS-WF increased lung PAFR mRNA expression at 24 hours (Fig 8). Pretreatment of MS-WF– exposed mice with CV-3988 1 hour before infection attenuated BALF CFU values. Pretreatment of MS-WF–exposed mice with CV-3988 did not reduce lung CFU values (Fig 9). In PBS-treated animals CV-3988 had no effect on either BALF or lung CFU values (data not shown).

PAFR: Stored samples

A 10-day course of 40 mg/m^3 of aerosolized StS-WF for 3 hours per day increased mouse lung PAFR mRNA expression compared with that seen in air-exposed control subjects at both 4 hours and 28 days after the last dose (see Fig E4 in this article's Online Repository at www.jacionline.org).

Lung biopsy tissue was available from a single nonsmoking welder and a single nonsmoking control subject. Specific PAFR immunostaining of bronchial and alveolar epithelial cells was present in the nonsmoking welder. Less intense specific bronchial epithelial PAFR was present in the nonsmoking control subject (see Fig E5 in this article's Online Repository at www.jacionline.org).

DISCUSSION

In this study we sought to identify a mechanism for the hypersusceptibility of welders to bacterial pneumonia reported in epidemiologic studies.[2,4,24] We focused on *S pneumoniae* because this bacterium is the most common cause of community-acquired pneumonia in adults.[10] In addition, a review of all patients presenting with invasive pneumococcal disease (IPD) in Alberta (Canada) from 2000 to 2004 by Wong et al[25] reported a 2.7-fold greater incidence of IPD in welders; of the 18 welders with IPD, 17 had bacteremic pneumococcal pneumonia, 1 had meningitis, and 1 died of pneumococcal infection. Pneumococcal infection in welders remains a problem. For example, in April 2015, the Northern Ireland

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Health Protection Service investigated an outbreak of IPD in shipyard workers and identified WF exposure as a possible risk factor.[26] In the present study we found that intranasal instillation of MS-WF in mice, followed by infection with *S pneumoniae*, resulted in a 50- to 175-fold increase in airway and lung CFU values.

We also found that MS-WF–induced hypersusceptibility to pneumococcal infection is mediated in part by PAFR (a host receptor used by pneumococci to adhere to and infect lower airway cells[13]) because MS-WF increased mouse lung PAFR mRNA expression and that treatment of mice with the PAFR blocker CV-3988 before pneumococcal infection significantly reduced lower airway bacterial load. Additional evidence for a role of PAFR was provided by *in vitro* experiments. First, MS-WF stimulated PAFR-dependent adhesion and infection of human lower airway cells. Second, CV-3988 attenuated MS-WF–stimulated pneumococcal adhesion and infection of human airway cell line cells and primary bronchial epithelial cells. Pneumococcal adhesion and infection stimulated by MS-WF is likely to be mediated by cellular oxidative stress because this is blocked by the antioxidant NAC. Indeed, these data are compatible with previous reports of induction of cellular oxidative stress by WF[27] and the capacity of NAC to attenuate pneumococcal adhesion and infection stimulated by fossil-fuel PM.[14] Furthermore, increased glutathione peroxidase and total antioxidants in the serum of active welders provides evidence that WF induce oxidative stress *in vivo*.[28]

To date, the role of PAFR in mediating vulnerability to pneumococcal pneumonia in human subjects is not fully defined.[29] However, this role is well established in animal models. For example, reduced PAFR expression by lower airway epithelial cells decreases mortality from pneumococcal infection in mice.[30] Conversely, increased airway epithelial PAFR causes hypersusceptibility of mice to pneumococcal infection.[31] Indirect evidence that airway PAFR is important in human subjects is provided by our previous observation that bronchial epithelial PAFR expression is increased in smokers.[15] In the present study PAFR was more strongly expressed by lower airway epithelial cell counts in the nonsmoking welder compared with the nonsmoking, non–WF-exposed control subject. To determine whether epithelial PAFR expression is increased in welders requires further lung biopsy samples from nonsmoking welders and nonsmoking control subjects, but to date, these have not been obtained.

The limitations of this study are as follows. First, it is unclear whether the concentrations of MS-WF used *in vitro* reflect exposure of airway cells *in vivo*. However, concentrations of MS-WF used in the adhesion and infection assays are similar to those estimated by Phalen et al[32] (85 μg/cm^2) for hot spots of inhaled PM deposition on airway cells. Furthermore, we found that lower concentrations of MS-WF (ie, 5 μg/cm^2) stimulated pneumococcal adhesion and infection but required prolonged culture duration.

Second, it is unclear whether a single 600 μg intranasal dose of MS-WF in the mouse reflects the dose inhaled by welders. However, there is evidence that welders inhale very high concentrations of PM. For example, Kim et al[33] reported mean daily exposures of welders to inhalable PM of 1660 μg/m^3 compared with 40 μg/m^3 in nonexposed control subjects.

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Third, we did not assess the effect of aerosolized MS-WF, amore physiologic delivery method. However, using stored samples, we found that exposure of mice to aerosolized StS-WF stimulates lung PAFR mRNA expression. Furthermore, our pilot data suggest that StS-WF also stimulates PAFR-dependent pneumococcal adhesion and infection to lower airway cells *in vitro* (see Fig E6 in this article's Online Repository at www.jacionline.org). Finally, it is unclear why pretreatment of MS-WF–exposed mice with the PAFR blocker CV-3988 attenuates airway (BALF) CFU values but does not attenuate lung tissue CFU values. We speculate that although PAFR-dependent adhesion is important in establishing airway infection, PAFR-independent mechanisms contribute to the development of pneumococcal infection in the lung tissue compartment.

In summary, we found that MS-WF increases PAFR-dependent pneumococcal adhesion and infection of human lower airway cells *in vitro* and pneumococcal airway infection in mice. This study suggests a mechanism for the increased vulnerability of welders to pneumococcal pneumonia reported in epidemiologic studies. Therefore these data provide biological plausibility for the UK Health and Safety Executive Guideline that the 23-valent pneumococcal polysaccharide vaccine "should be considered for people whose work exposes them to frequent or continuous exposure to metal fume (e.g. welders), taking into account the exposure control measures in place."[34]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BALF	Bronchoalveolar lavage fluid
CFU	Colony-forming units
IPD	Invasive pneumococcal disease
LDH	Lactate dehydrogenase
MS-WF	Mild steel welding fumes
NAC	N-acetylcysteine
OP	Oxidative potential
PAFR	Platelet-activating factor receptor

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PM	Particulate matter
StS-WF	Stainless steel welding fumes
UK	United Kingdom
WF	Welding fumes

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Key messages

- Exposure of human lower airway epithelial cells to WF in vitro results in hypersusceptibility to platelet-activating factor–dependent pneumococcal adhesion and infection.
- Exposure of mice to WF results in hypersusceptibility to pneumococcal airway infection.
- The increased risk of pneumococcal pneumonia in welders reported in epidemiologic studies is biologically plausible.

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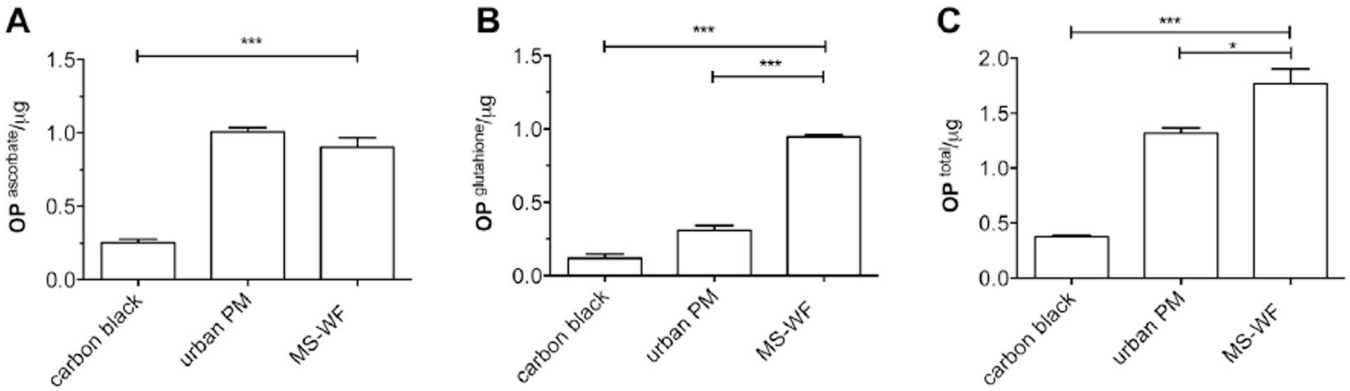

FIG 1.

The OP of MS-WF assessed based on their *in vitro* capacity to deplete antioxidants over 4 hours. Particle standards included in the assay are as follows: (1) low-OP carbon black (M120) and (2) high-OP urban air PM (NIST1648a). The OP of MS-WF (OP per microgram of PM) is given for ascorbate (**A**), glutathione (**B**), and total values (**C**). Data are from 3 experiments and presented as means (SEMs). Comparisons are performed by using 1-way ANOVA with the Tukey *post hoc* multiple comparison test. *$P < .05$ and ***$P < .001$.

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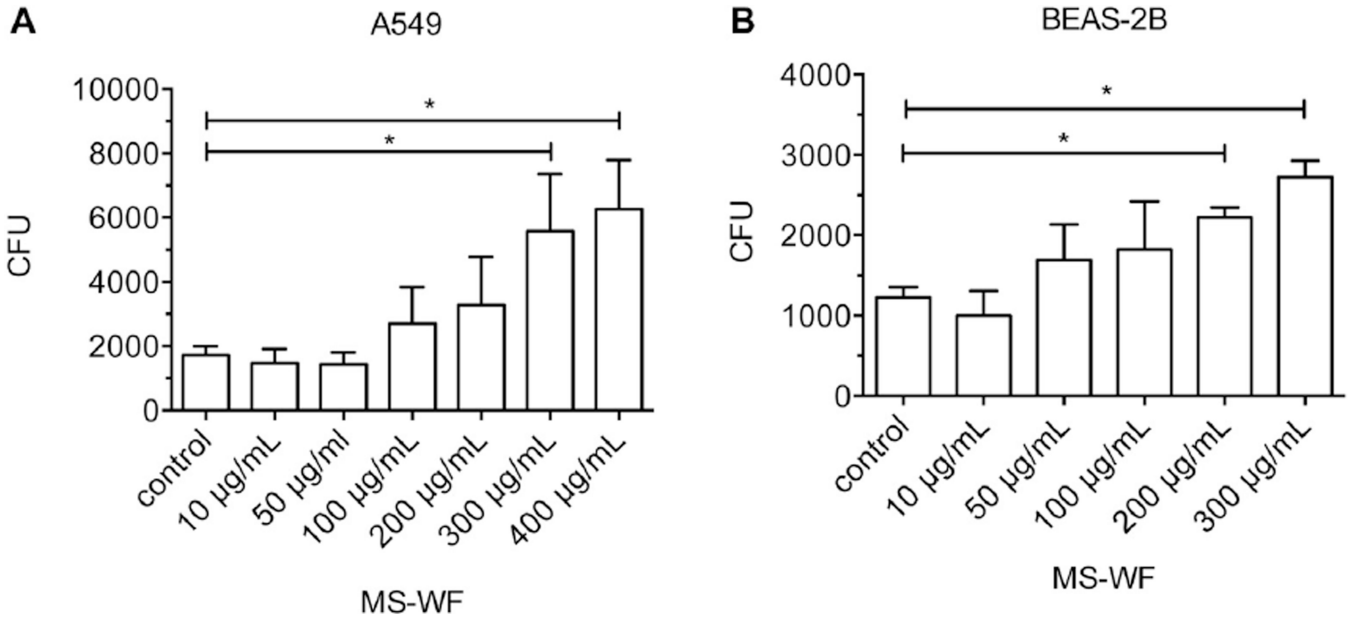

FIG 2.

Effect of 2 hours of exposure of human airway cells *in vitro* to MS-WF on pneumococcal adhesion and infection. Cells were infected with *S pneumoniae* for 2 hours at a multiplicity of infection of 100. **A**, A549 cells. **B**, BEAS-2B cells. Increased pneumococcal adhesion and infection are reflected by increased CFU values determined by means of quantitative bacterial culture. Data are from 3 to 4 separate experiments, each with 3 technical replicates, and presented as means (SEMs). Data are compared by using 1-way ANOVA and the Tukey *post hoc* multiple comparison test. *$P < .05$.

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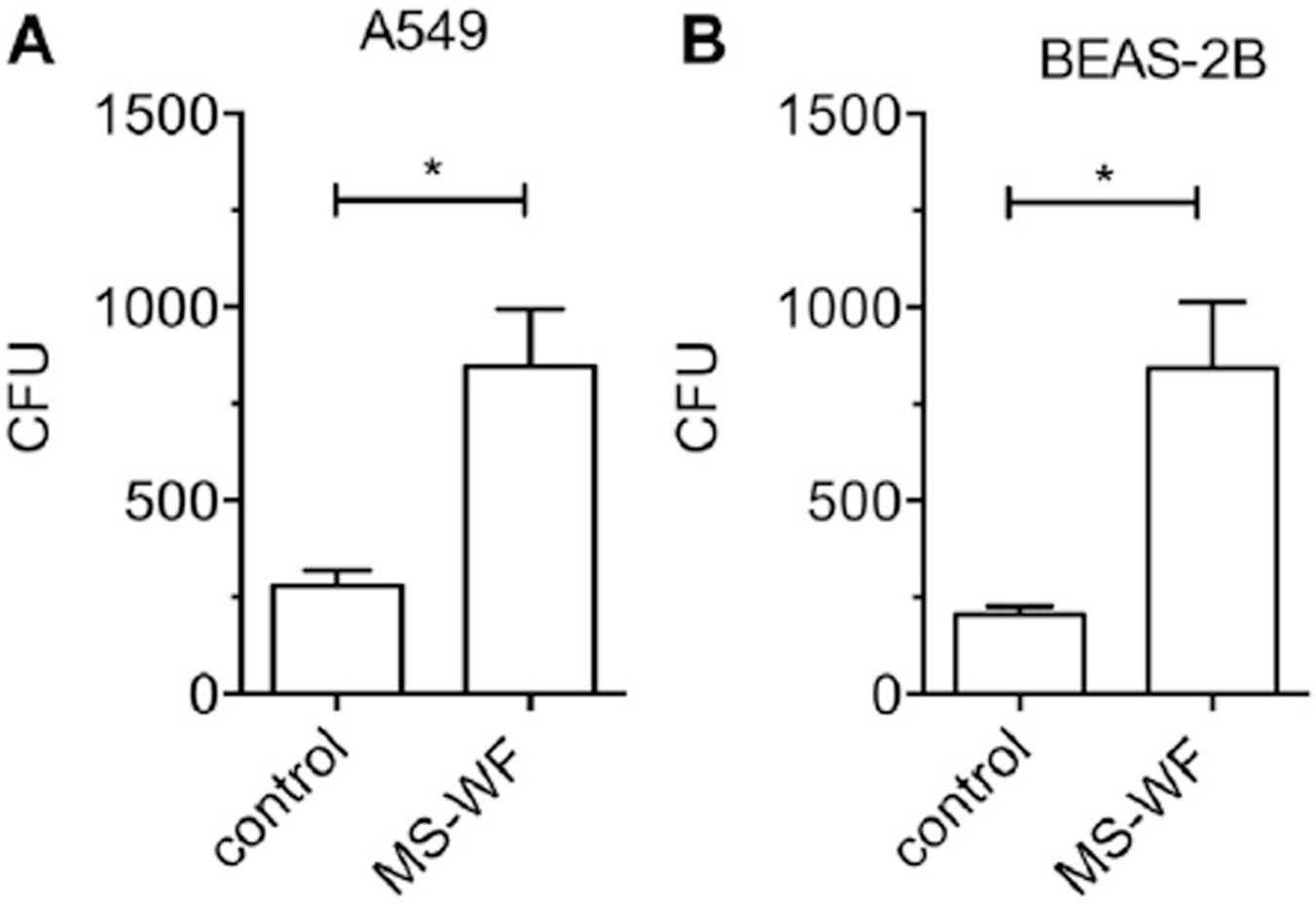

FIG 3.

Effect of exposure of human airway cells *in vitro* to MS-WF for 2 hours on the infective fraction of *S pneumoniae*. Cells were infected with *S pneumoniae* for 2 hours at a multiplicity of infection of 100. Pneumococci that were adherent to cell surfaces were first killed by antibiotics to assess the infective fraction. Intracellular bacteria that were protected from antibiotics were then recovered by means of cell lysis, and CFU values were assessed by means of quantitative culture. **A**, A549 cells cultured with 275 μg/mL (145 μg/cm^2). **B**, BEAS-2B cells cultured with 200 μg/mL (105 μg/ cm^2). Data are from 3 separate experiments, with 3 technical replicates per experiment, and presented as means (SEMs). Data are compared by using *t* tests. *$P < .05$.

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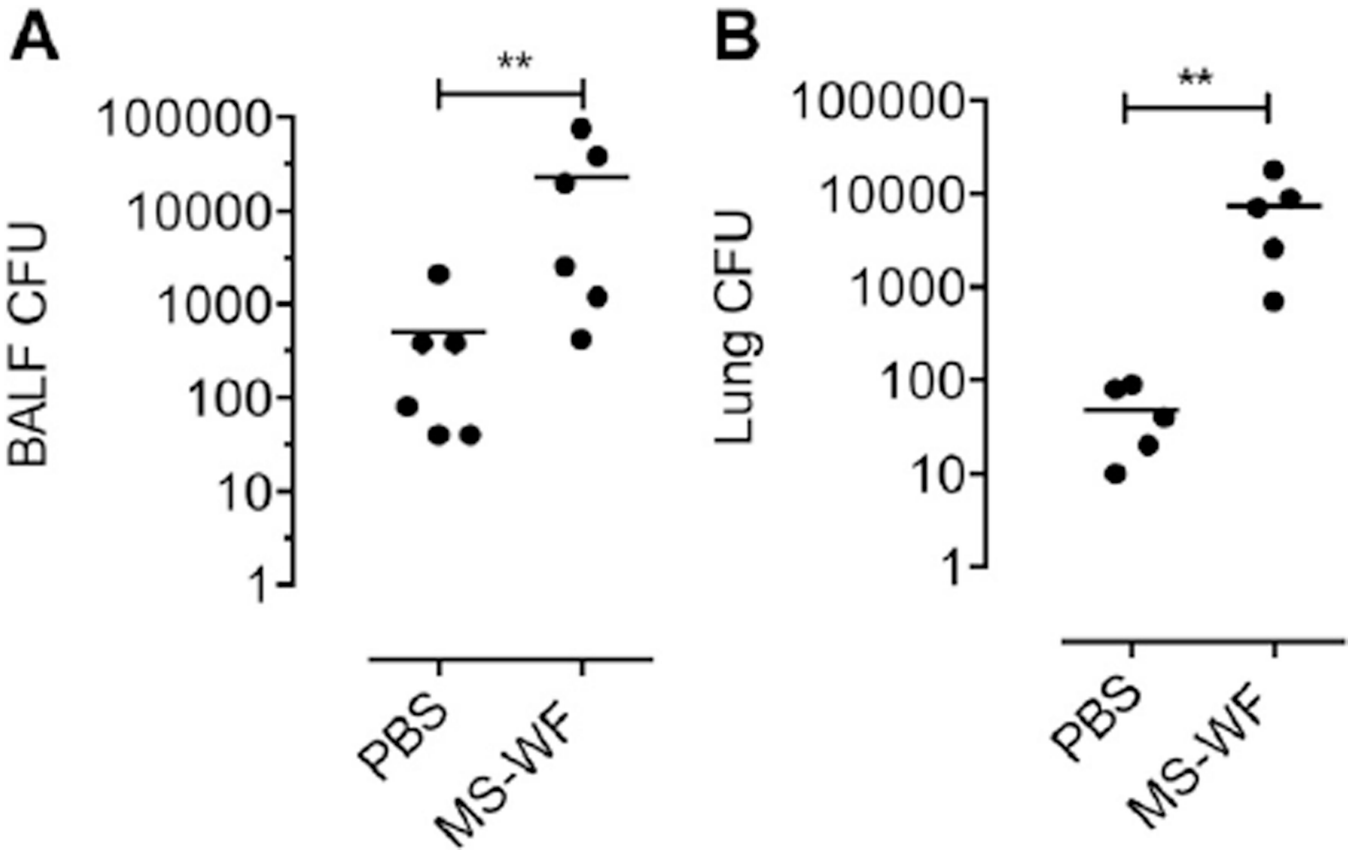

FIG 4.

Effect of exposure of mice to a single 600 μg intranasal dose of MS-WF on *S pneumoniae* CFU values. Mice were infected 24 hours after instillation of MS-WF, and CFU values were assessed by means of qualitative culture 24 hours after infection. **A**, BALF CFU values. **B**, Lung tissue CFU values. Dot plots are from 6 animals per group and compared by using the Mann-Whitney *U* test. *Bars* represent medians. $^{**}P < .01$.

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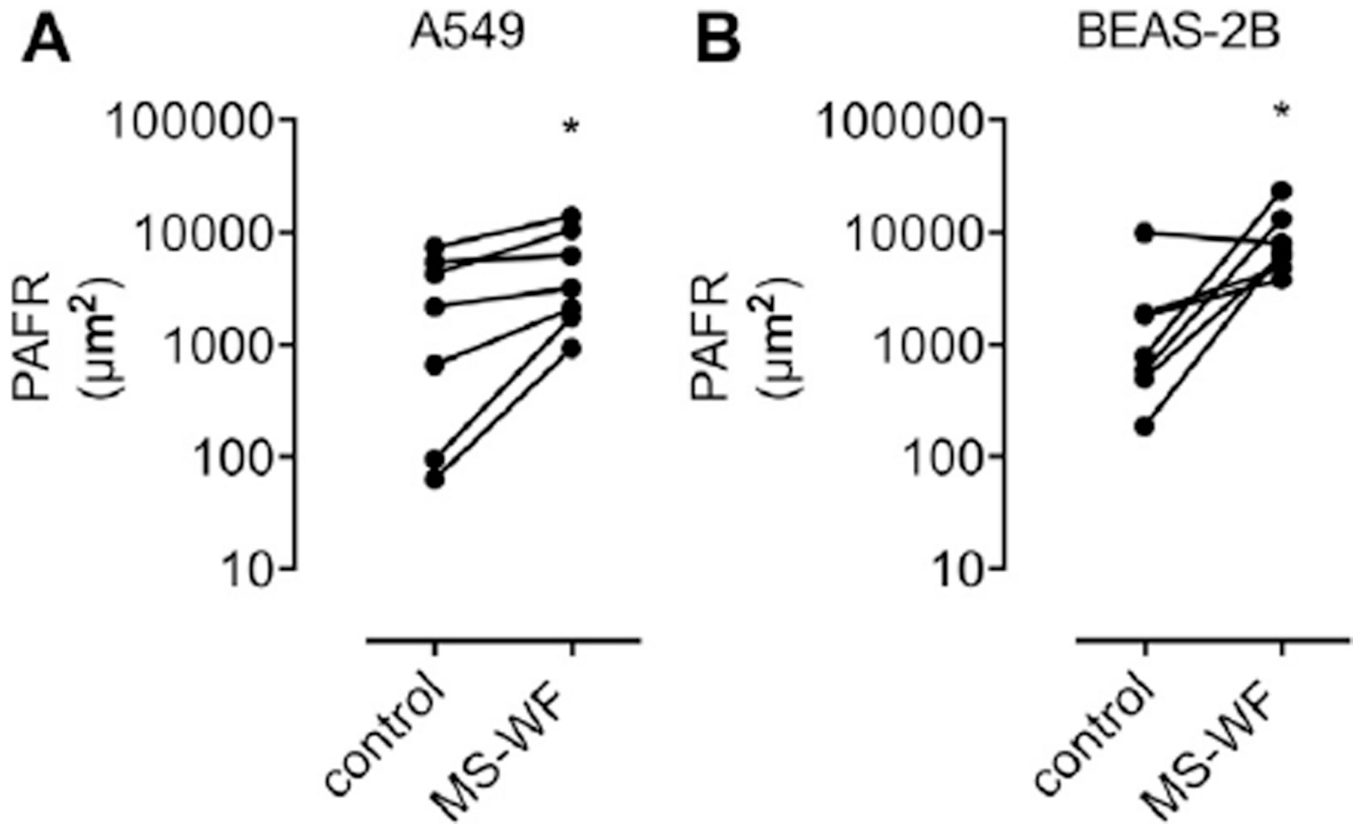

FIG 5.

Effect of exposure of MS-WF on PAFR protein expression by human airway cells. Images were taken by using an epiflorescence microscope and analyzed with ImageJ software. A florescence intensity threshold was set to discount background florescence. The area of florescence (in square micrometers) was then measured for each image. **A**, A549 cells cultured with MS-WF (275 μg/mL) for 2 hours. **B**, BEAS-2B cells cultured with MS-WF (200 μg/mL) for 2 hours. Data are from 3 to 4 separate experiments, with 3 replicates per experiments. Control PAFR expression in separate experiments is highly variable, and data are therefore compared by using paired *t* tests. *$P < .05$ versus control subjects.

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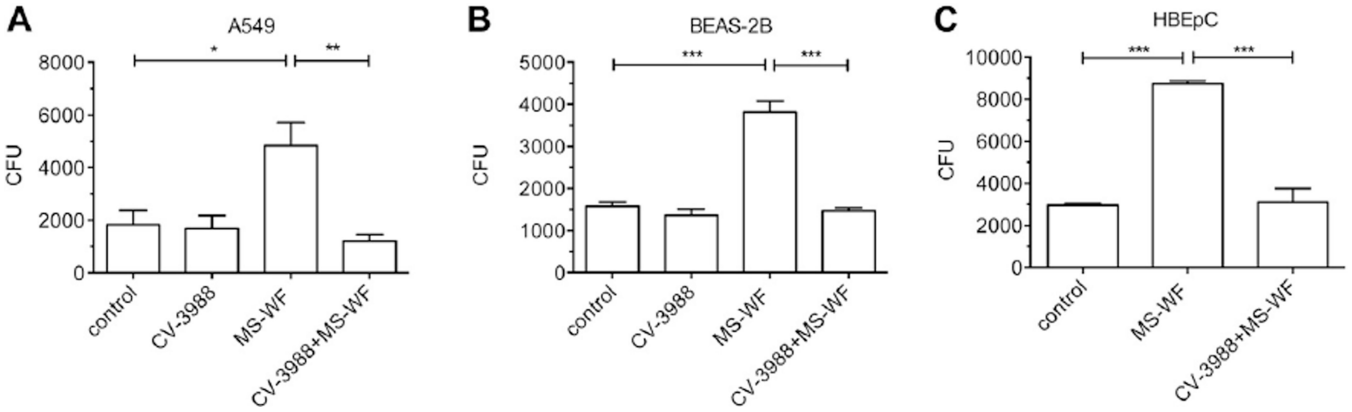

FIG 6.

Effect of the PAFR blocker CV-3988 (20 μmol/L) on *S pneumoniae* adhesion and infection to A549 cells cultured with MS-WF (275 μg/mL; **A**), BEAS-2B cells cultured with MS-WF (200 μg/mL) for 2 hours (**B**), and human primary bronchial epithelial cells cultured with MS-WF (200 μg/mL) for 2 hours (**C**).Data are from3 ormore separate experiments, with 3 replicates per experiment, and presented as means (SEMs). Data are compared by using 1-way ANOVA and the Tukey multiple comparison test. *$P < .05$, **$P < .01$, and ***$P < .001$.

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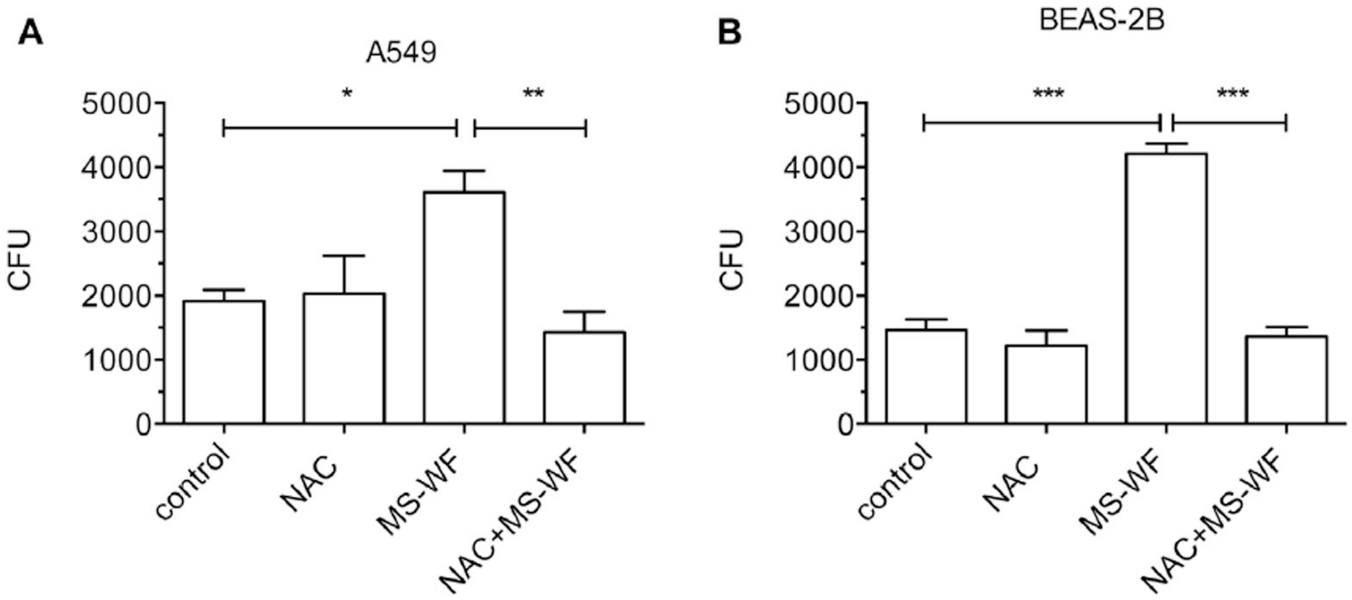

FIG 7.

Effect of the antioxidant NAC on adhesion and infection of *S pneumoniae* to airway cells. NAC was added to cells at the same time as MS-WF. **A**, A549 cells cultured with MS-WF (275 μg/mL) for 2 hours. **B**, BEAS-2B cells cultured with MS-WF (200 μg/mL) for 2 hours. Data are from 3 separate experiments, with 3 technical replicates per experiment, and presented as means (SEMs). Data are compared by using 1-way ANOVA and the Tukey *post hoc* multiple comparison test. $*P < .05$, $**P < .01$, and $***P < .001$.

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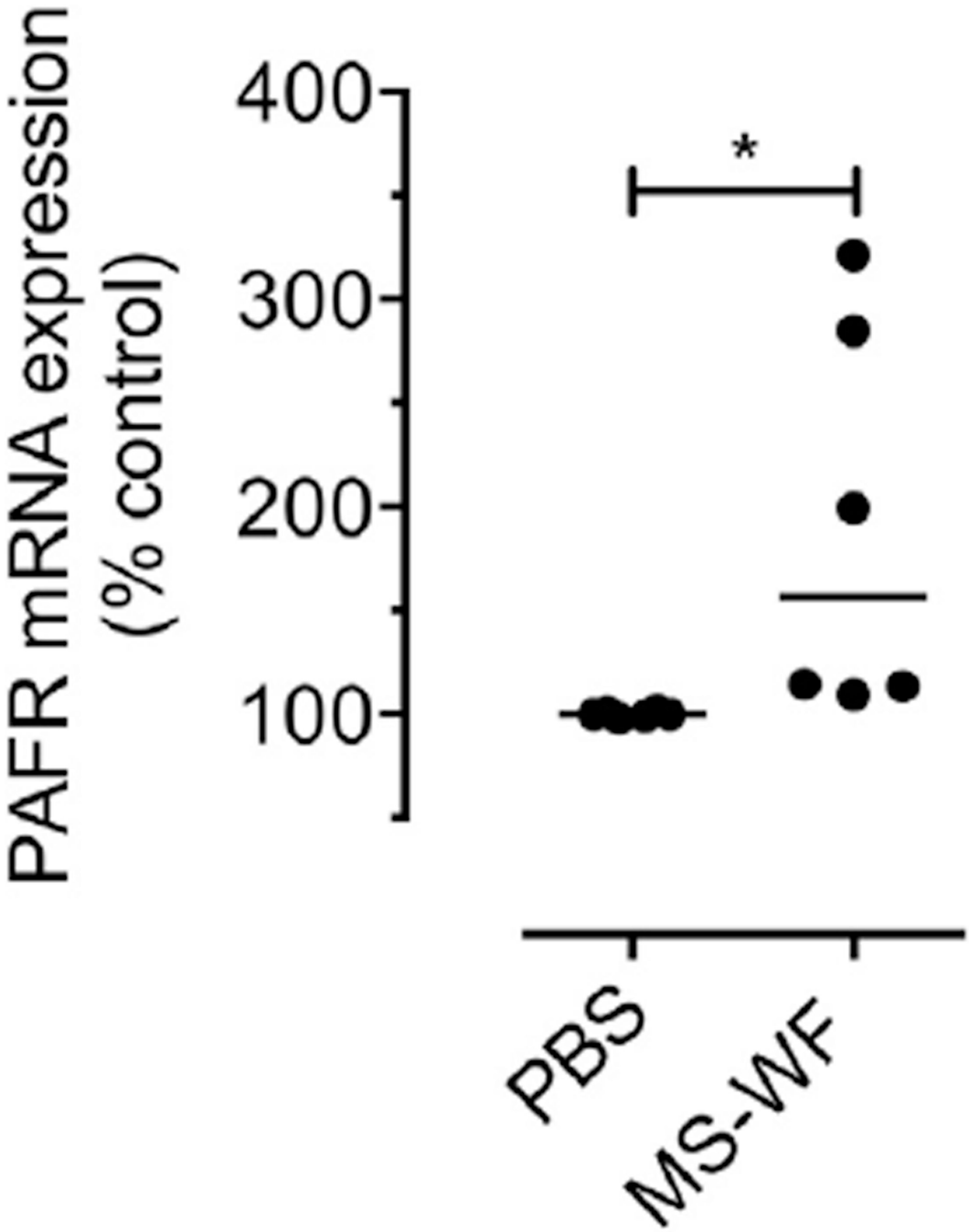

FIG 8.

Effect of a single 600 μg intranasal dose of MS-WF on mouse lung PAFR mRNA expression assessed at 24 hours after instillation. Lung PAFR mRNA expression was assessed by means of real-time PCR with relative quantification by using normalization to the reference gene β_2-microglobulin. Dot plots are from 6 mice per group and compared by using the Mann- Whitney test. The *bar* represents the median. *$P < .05$.

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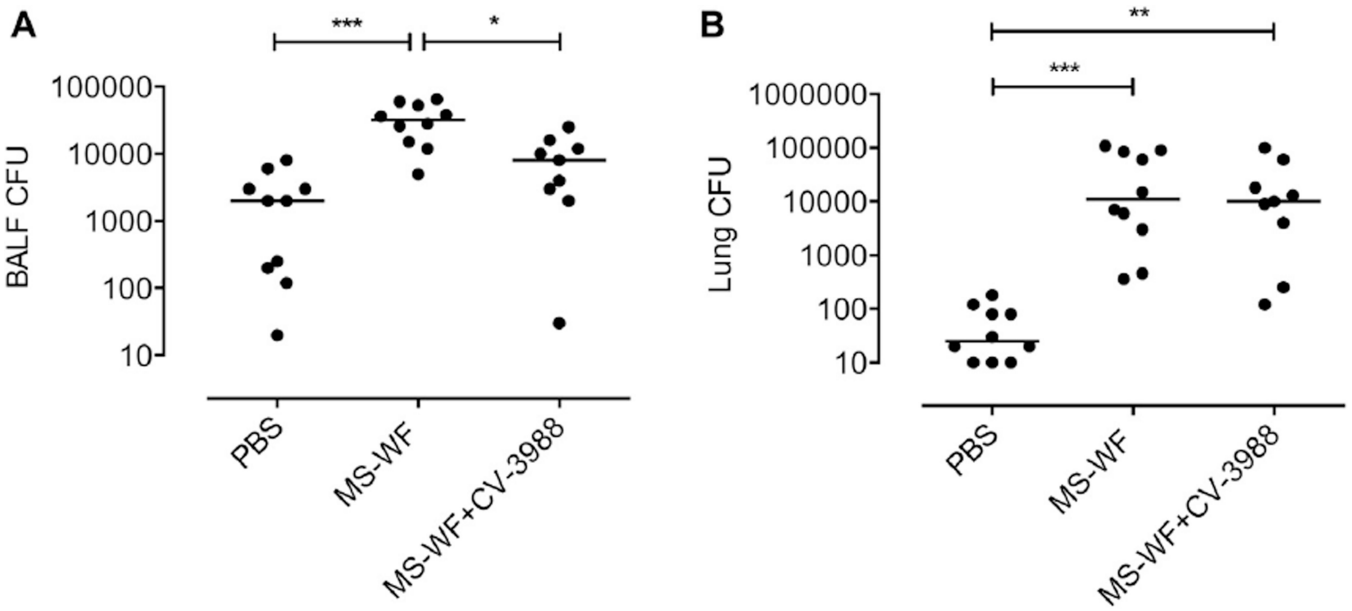

FIG 9.

Effect of intravenous treatment of mice with the PAFR blocker CV-3988 (5 mg/kg) administered 1 hour before infection with *S pneumoniae* in animals exposed to a single 600 μg intranasal dose of MS-WF. **A**, BALF pneumococcal CFU values. **B**, Lung pneumococcal CFU values. Data are representative of 2 separate experiments and compared by using the Kruskal-Wallis test and Dunn multiple comparison test. *Bars* represent medians. *$P < .05$, **$P < .01$, and ***$P < .001$.

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TABLE I

Composition of MS-WF

	Total weight of sample (%)
Aluminum	0.3
Barium	<0.1
Bismuth	<0.1
Calcium	8.9
Cobalt	<0.1
Chromium	<0.1
Copper	<0.1
Iron	12.4
Potassium	23.6
Lithium	0.4
Magnesium	5.3
Manganese	3.8
Molybdenum	<0.1
Sodium	2.6
Nickel	<0.1
Lead	<0.1
Silicon	2.1
Titanium	0.6
Vanadium	<0.1
Zinc	0.2
Fluoride ions	17.9
Chromium (VI)	<0.1

Each element is represented as a percentage of the total weight of the sample provided. The composition of MS-WF was determined by using inductively coupled plasma–atomic emission spectroscopy.