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RESEARCH PAPER

Cigarette smoke inhibits macrophage sensing of Gram-negative bacteria and lipopolysaccharide: relative roles of nicotine and oxidant stress

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Background and purpose: Smoking cigarettes is a major risk factor for the development of cardiovascular and respiratory disease. Moreover, smokers are more prone to infections. This has been associated with a suppression of the immune system by smoke. However, it is not clear how cigarette smoke affects the ability of immune cells to sense pathogens. Cigarette smoke contains a large number of molecules which may mediate responses on immune cells and of these, nicotine and oxidants have both been identified as inhibitory for the sensing of bacterial lipopolysaccharide (LPS). Nitric oxide synthase (NOS) and tumour necrosis factor (TNF)-α are both induced in macrophages on stimulation with Gram negative bacteria or LPS.
Experimental approach: We used murine macrophages stimulated with whole heat-killed bacteria or LPS. We measured output of NO (as nitrite) and TNFα, NOS protein by Western blotting and cellular oxidant stress.
Key results: Cigarette smoke extract suppressed the ability of murine macrophages to release NO, but not TNFα in response to whole bacteria. Cigarette smoke extract also inhibited nitric oxide synthase II protein expression in response to LPS. The effects of cigarette smoke extract on nitrite formation stimulated by LPS were unaffected by inhibition of nicotinic receptors with α-bungarotoxin (100 units ml^{-1}). However, the effects of cigarette smoke extract on LPS-induced nitrite formation were mimicked by hydrogen peroxide and reversed by the anti-oxidants N-acetyl cysteine and glutathione.
Conclusions and implications: We suggest that cigarette smoke exerts its immunosuppressive effects through an oxidant-dependent and not a nicotine-dependent mechanism.
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Keywords: nitric oxide; LPS; cigarette smoke; chronic obstructive pulmonary disease; Toll-like receptor; nicotine

Abbreviations: COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; LPS, lipopolysaccharide

Introduction

Smoking cigarettes increases the risk of premature death from heart attack, stroke, chronic obstructive pulmonary disease (COPD) and cancer (Sloan *et al*., 2004). It is now recognized that smoking cigarettes can also increase a smoker's chances of dying from an infection (Bartal, 2001). Cigarette smoke can have direct inhibitory effects on the sensing of bacterial 'pathogen-associated molecular patterns' such as bacterial lipopolysaccharide (LPS) *in vitro* (Laan *et al*., 2004). However, the mechanism by which cigarette smoke inhibits the sensing of LPS by macrophages is unknown. Moreover, the effect of cigarette smoke on the activation of macrophages by whole bacteria has not previously been investigated.

Therefore, in the current study, we have used whole Gram-positive *Staphylococcus aureus* or *Streptococcus pneumoniae* or Gram-negative *Escherichia coli* or *Pseudomonas aeruginosa* to activate macrophages and investigated the effects of cigarette smoke on associated responses. NOSII and tumour necrosis factor (TNF)-α are proteins central to the processes of innate immunity and the corresponding genes are co-induced by bacteria in macrophages (Paul-Clark *et al*., 2006). We have therefore measured nitric oxide (NO) and TNFα as markers of cell activation in this study.

Cigarette smoke contains a large number of biologically active molecules. However, the ability of cigarette smoke to

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cause oxidant stress in cells is likely to be important in any biological response observed in cells *in vitro*. In addition, cigarette smoke contains nicotine, which has recently been shown to inhibit the ability of macrophages to sense LPS *in vitro* (Borovikova *et al*., 2000). The hypothesis that we have been working towards is that cigarette smoke induces inflammation and disease via oxidant stress-dependent mechanisms and that this has important implications for innate immune responses. Thus, in the current study, we have specifically investigated the role of oxidant stress and nicotine-like components of smoke on activation of macrophages by whole bacteria.

Methods

Test systems used

Bacterial culture and preparation. All bacteria were stored as frozen glycerol stocks and streaked onto agar plates before inoculation of single colonies into RPMI medium 1640 with 10% fetal calf serum and glutamine. Cultures were incubated overnight and then centrifuged at 850 *g* to pellet bacteria. Bacteria were then washed twice in sterile saline, and pellets were resuspended in sterile saline. Aliquots of the bacterial suspension were serially diluted and plated onto agar to quantify bacterial cell numbers. The bacterial suspensions were then heat-treated for 60 min at 80 °C to kill all bacteria; sterility was confirmed by plating of the resultant suspension. An aliquot of the live bacterial suspension was plated onto agar for 24 h and colonies were counted for the estimation of final colony-forming units (CFU) counts. Suspensions of the bacteria were adjusted to 10^{10}–10^{12} CFU equivalents per ml and then frozen in 20% glycerol in aliquots before use in cell culture experiments. *E. coli* strain O111.B4, *S. aureus* strain H380, *S. pneumoniae* and *P. aeruginosa* were used throughout. Bacterial LPS from the *E. coli* strain O111.B4 was purchased from Sigma (Dorset, UK).

Cell culture. J774.2A murine macrophages were purchased from the European Cell Collection (Sailsbury, Wiltshire, UK). Primary cultures of murine macrophages derived from bone marrow were isolated from femurs and differentiated as described previously (Paul-Clark *et al*., 2006). Cells were cultured in Dulbecco's modified Eagle's medium containing 1 mM sodium pyruvate and phenol red, supplemented with penicillin (100 U ml^{-1}), streptomycin (1 μg ml^{-1}), L-glutamine (2 mM), amphotericin B (2.5 μg ml^{-1}), a mixture of non-essential amino acids and 10% fetal calf serum. Cells were maintained in a humidified incubator at 37 °C, 5% CO_2 and 95% air.

Cells were plated on 96-well plates at a density of 5×10^5 ml^{-1} (1×10^5 cells per well). After plating, the cells were grown to confluence. Medium was then replaced and cells treated appropriately.

Preparation of smoke solutions. All cigarettes used in this study were Marlboro Red cigarettes (Tar 11 mg, nicotine 0.8 mg, carbon monoxide 11 mg; Philip Morris International-Ausanne, Switzerland). Cigarette smoke extracts (CSEs) were made as described previously, using the smoke from four cigarettes in 100 ml (Walters *et al*., 2005) of serum-free culture medium. The prepared CSE solution was filtered through a 2 μm microfilter and used immediately due to its labile nature (Walters *et al*., 2005). This solution was described as '100%' and standardized by measuring nitrite concentration, as we have described previously (Walters *et al*., 2005). In other studies using CSE, the 'strength' of preparation is often assessed by measuring absorbance of solutions at 320 nm (Mio *et al*., 1997; Yang *et al*., 2006). In the current study, we have included data that show how the strength of our CSE preparations compares with others. We have also included data to show the oxidant potential of our smoke solutions by measuring its ability to convert dihydrorhodamine-123 to rhodamine-123 (Figure 8) (see also below for details of how this assay was used to assess oxidant changes within cells).

Measurements made

Measurement of NO production. NO production by cells was measured by the accumulation of its oxidation product, nitrite using the Griess reaction, as we have described previously (Paul-Clark *et al*., 2006). Briefly, 100 μl of cell-free supernatant was mixed with equal proportions of Griess reagent (sulphanilamide 0.5%, orthophosphoric acid 2.5 and 0.05% *N*-(1-naphthyl) ethylenediamine). Nitrite levels were measured at 550 nm and results were expressed as micromolar concentrations.

Determination of cell viability. The effect of treatments on cell viability was indexed by measuring respiration, as we have shown previously (Paul-Clark *et al*., 2006). Respiration was assessed by measuring the mitochondrial-dependent reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, Poole, UK) to its formazan dye. This was performed following all treatments. Unless otherwise stated, none of the treatments affected MTT conversion by cells.

ELISA for TNFα. TNFα levels in cell-free supernatant were determined by ELISA using commercially available matched antibody pairs following a protocol furnished by the manufacturer (R&D Systems, Abingdon, UK). TNFα concentrations were measured at 450 nm with a reference filter at 550 nm and results expressed as ng ml^{-1}.

Western blot analysis. NOSII was measured in cells by western blotting, as we have described previously (Paul-Clark *et al*., 2006). In brief, cells were plated into six-well culture plates and treated with LPS with or without CSE for 24 h. The medium was removed and cells were washed twice with ice-cold PBS. Cells were lysed using HEPES (10 mM) containing $MgCl_2$ (3 mM), KCl (40 mM), glycerol (5%), Nonidet P-40 (0.3%) and phenylmethanesulphonyl fluoride (1 mM). Protein concentration in whole-cell preparations was measured using the Bradford assay. Samples were separated by gel electrophoresis on 7.5% sodium dodecyl sulphate-polyacrylamide gels; after transfer onto nitrocellulose membranes, NOSII was detected using a specific polyclonal rabbit antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA,

USA) and the signal was amplified with goat anti-rabbit IgG-horseradish peroxidase (1:1000; Dako, Cambridgeshire, UK). Blots were visualized onto film using electrochemiluminescence reagents (Amersham Biotechnology, Oxford, UK). After the blots were stripped using Restore Western Blot Stripping Buffer (Pierce Biotechnology Inc., Rockford, IL, USA) and probed with β-actin (1:2000) that was used to confirm equal protein loading between lanes.

Measurement of oxidant stress. Oxidant stress in cells was measured by the conversion of dihydrorhodamine-123 to rhodamine-123, its oxidation product. Briefly, 100 μl of 0.5 μM dihydrorhodamine-123 in culture media was added to cells and incubated in a cell culture incubator for 30 min. The media were replaced and the stimulus (H_2O_2 (1 mM) or CSE) was added in fresh media for 10 min. Fluorescence was then measured in a plate reader (BioTek Instruments Inc., Winooski, Vermont, USA, Synergy HT) at 428 nm excitation and 535 nm absorbance. Concentrations of rhodamine-123 generated in cells were calculated using a standard curve of authentic rhodamine-123 (0.0131–4 μM).

Experimental design

Treating cells. Cells were treated with bacteria or LPS for 24 h before medium was removed for the measurement of NO or TNFα. In some experiments, cells were pretreated with CSE 20 min before the addition of bacteria or LPS. In other experiments, cells were pretreated for 30 min with *N*-acetylcysteine (0.01–1 mM), glutathione (0.01–1 mM) or α-bungarotoxin (1–100 U ml^{-1}) before the addition of CSE and/or LPS. In separate experiments, cells were treated with ACh (1–100 μM), nicotine (0.1–10 μM) or α-bungarotoxin (1–100 U ml^{-1}) alone or before the addition of CSE with or without LPS. Supernatants were either used immediately for nitrite determination or stored at −80 °C for the measurement of TNFα levels.

Data analysis and statistical procedures

All data are the mean ± s.e.mean from at least three experiments. Details of the numbers of assays and statistical tests performed are contained in the relevant figure legends.

Drugs, chemicals reagents and other materials

All drugs were obtained from Sigma Chemical Company (Poole, Dorset, UK), unless otherwise stated.

Results

Effects of Gram-negative bacteria on NOS and TNFα induction

Under control culture conditions, murine macrophages released low or undetectable levels of nitrite and TNFα. However, two species of Gram-negative bacteria, *E. coli* or *P. aeruginosa*, stimulated the cells to release NO, resulting in elevated levels of nitrite or TNFα in a concentration-dependent manner (Figure 1). In our hands, the effects of whole Gram-negative *P. aeruginosa*, at the highest concentrations tested, caused a small, but significant reduction in cell viability (data not shown).

Effects of Gram-positive bacteria on NOS and TNFα induction

In contrast to the results obtained using Gram-negative bacteria, Gram-positive *S. aureus* or *S. pneumoniae* did not induce statistically significant (one-way ANOVA $P>0.05$) increases in nitrite levels. However, both Gram-positive pathogens induced TNFα release from the cells with an efficacy similar to that seen with Gram-negative pathogens (Table 1). At the highest concentration used, *S. pneumoniae* (10^8 CFU ml^{-1}) reduced cell viability (data not shown).

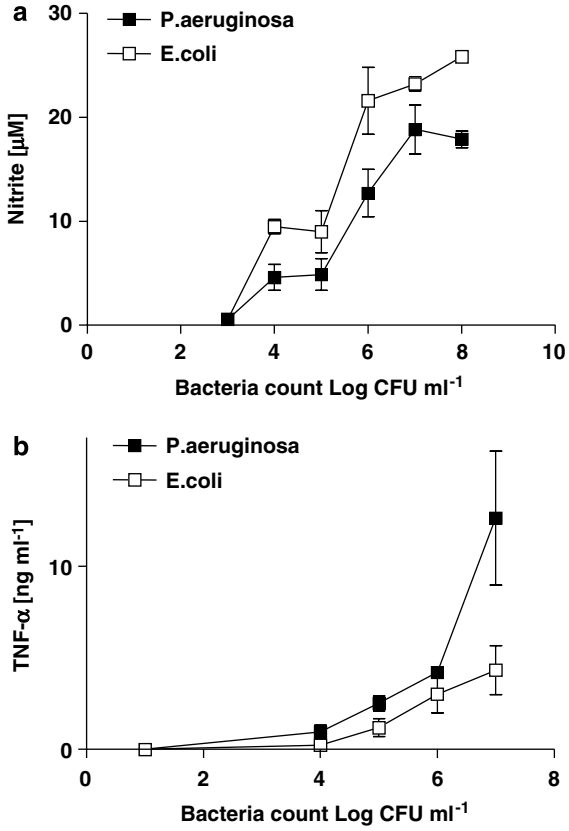

Figure 1 Effect of Gram-negative *P. aeruginosa* and *E. coli* on nitrite and TNFα formation by J774.2 macrophages. A range of concentrations of *P. aeruginosa* or *E. coli* were added to the cells for 24 h and (**a**) nitrite and (**b**) TNFα levels were measured. The data are the mean ± s.e.mean; $n=12$.

Table 1 Effect of Gram-negative and Gram-positive bacteria on NO (nitrite) and TNFα levels in J774.2 macrophages

Bacteria	*NO eMax (μM)*	*TNFα eMax (ng ml^{-1})*
Control	ND	ND
Escherichia coli Gram −ve	18.9 ± 2.4	4.3 ± 1.3
Pseudomonas aeruginosa Gram −ve	25.9 ± 0.3	4.2 ± 0.5
Staphylococcus aureus Gram +ve	4.7 ± 1.6	5.7 ± 1.2
Streptococcus pneumoniae Gram +ve	4.7 ± 0.5	3.5 ± 1.4

ND, not detectable; NO, nitric oxide; TNFα, tumour necrosis factor.
Mean eMax (maximum possible effect) values with s.e.mean for NO and TNFα release following stimulation with Gram-negative bacteria (*E. coli* and *P. aeruginosa*) or Gram-positive bacteria (*S. aureus* and *S. pneumoniae*) by J774.2 macrophages ($n=12$), below limits of assay; for NO = 1 μM; for TNFα = 15.6 pg ml^{-1}.

Effects of CSE on NO release induced by LPS or bacteria

The increased level of nitrite induced by Gram-negative *E. coli* was inhibited in a concentration-dependent manner by CSE, with complete abolition achieved with a 5% dilution of the CSE (Figure 2). CSE also inhibited nitrite formation by cells treated with *P. aeruginosa* (Figure 2). Similarly, CSE caused a concentration-dependent inhibition of nitrite formation induced by LPS ($1\,\mu g\,ml^{-1}$) (Figure 2). The inhibitory effect of CSE on nitrite formation was not due to any direct effect on the degradation of NO to higher oxides of nitrogen because it had no effect on nitrite accumulated (via free NO) following incubation of sodium nitroprusside (data not shown).

Effect of CSE on NOSII induction in macrophages

The increased levels of nitrite induced by either LPS or Gram-negative bacteria was inhibited by the selective NOSII inhibitor 1400W (10 μM; data not shown) and was associated with increased expression of NOSII immunoreactivity (Figure 3). The inhibitory effects of CSE on nitrite levels were paralleled by a reduction in the expression of NOSII protein (Figure 3).

Effects of CSE on TNFα release induced by Gram-negative or Gram-positive bacteria

In contrast to the effects of CSE on increased nitrite levels and NOSII induction, CSE had no significant effect on TNFα

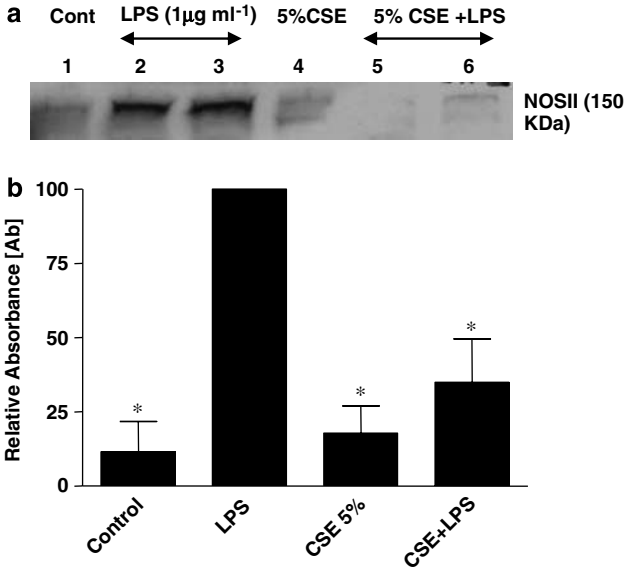

Figure 3 Effect of cigarette smoke extract (CSE) on NOSII protein expression. (**a**) A typical western blot for NOSII expression in J774.2 macrophages stimulated with LPS ($1\,\mu g\,ml^{-1}$) for 24 h in the presence or absence of CSE (5%). (**b**) Pooled data of the relative absorbance from four separate western blots. The data are normalized to the absorbance seen in cells treated with LPS (100%). The data were analysed using a one-sample *t*-test for normalized data. *$P<0.05$ vs LPS alone.

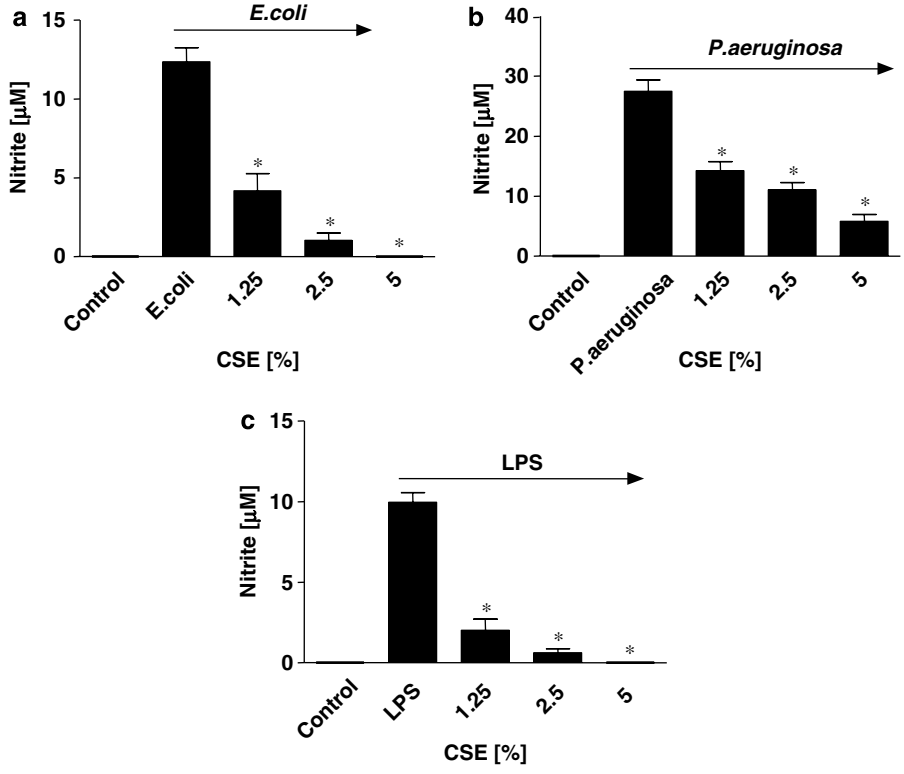

Figure 2 Effect of cigarette smoke extract (CSE) on nitrite formation induced by Gram-negative bacteria or LPS. (**a**) *E. coli* ($10^8\,CFU\,ml^{-1}$), (**b**) *P. aeruginosa* ($10^8\,CFU\,ml^{-1}$) or (**c**) LPS ($1\,\mu g\,ml^{-1}$) were added to J774 macrophages for 24 h in the presence or absence of CSE (1.25–5%). The data are the mean ± s.e.mean; $n=3$–9 experiments. Analysis was carried out using one-way ANOVA followed by a Bonferroni post-test. *$P<0.05$ vs stimulus alone.

release from macrophages activated by either *E. coli* or *S. aureus* (Figure 4). However, when LPS was used to activate cells, CSE significantly reduced TNFα release (LPS alone 4465 ± 1348 pg ml^{-1}, with 5% CSE 769 ± 309 ng ml^{-1}; $n=6$).

Role of nicotine in CSE-induced inhibition of NOSII induction by LPS in macrophages

LPS-induced nitrite release was unaffected by adding the muscarinic/nicotinic agonist ACh (1 μM), the nicotinic selective ligand nicotine (10 nM) or the selective nicotinic antagonist α-bungarotoxin (100 U ml^{-1}) to the incubations (Figure 5). Furthermore, the inhibitory effects of CSE on LPS-induced nitrite release were unaffected by α-bungarotoxin (Figure 5). As seen with the macrophage cell line (Figure 2), CSE inhibited LPS (1 μg ml^{-1}) induced increases in nitrite from primary cultures of murine bone marrow-derived macrophages (LPS, 23 ± 0.2 μM: plus 5% CSE below limit of detection >1 μM). Moreover, as was the case with J774 macrophages, nicotine (10 nM) had no effect on LPS-induced nitrite release by primary cultures of murine bone marrow-derived macrophages (LPS, 80 ± 1 μM: LPS plus nicotine, 78 ± 0.9 μM; $n=3$).

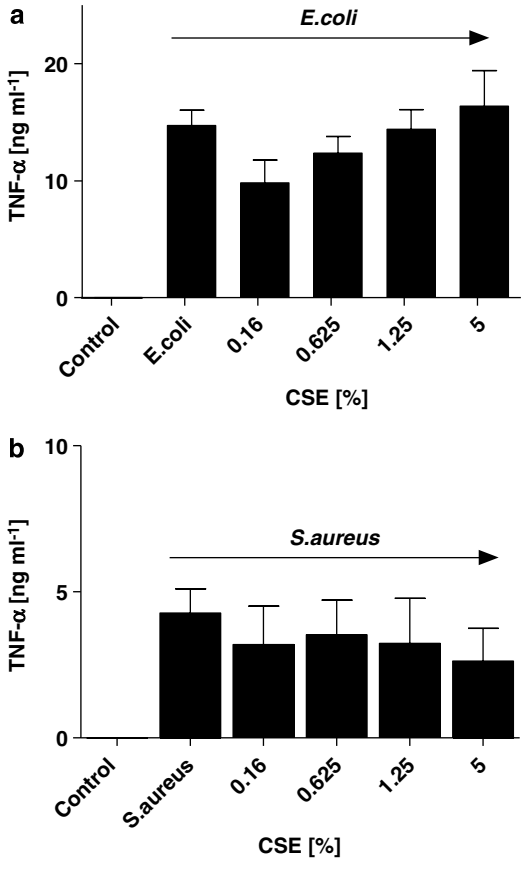

Figure 4 Effect of cigarette smoke extract (CSE) on TNFα release from cells stimulated with (**a**) *E. coli* or (**b**) *S. aureus.* Bacteria (10^8 CFU ml^{-1}) in the presence or absence of CSE (0.16–5%) were added to cells for 24 h. The data are the mean ± s.e.mean; $n=5$–9 experiments. Analysis was carried out using one-way ANOVA followed by a Bonferroni post-test.

Role of oxidant stress in CSE-induced inhibition of nitrite release by J774 macrophages

Exposure of cells to CSE caused a detectable oxidant stress measured by the formation of free rhodamine-123 (Figure 6). Pretreatment of cells with the antioxidant *N*-acetylcysteine (0.01–1 mM) resulted in a concentration-dependent reversal of the inhibitory effect of CSE (2.5%) on increased levels of nitrite from cells activated by LPS (Figure 6). In separate experiments, another antioxidant glutathione (1 mM) partly reversed the inhibitory effect of CSE (2.5%) on LPS-induced NO release (control, below 1 μM; plus LPS, 7 ± 1.6 μM; LPS plus CSE, 1.6 ± 0.3 μM; LPS + CSE + glutathione, 4.2 ± 1.2 μM, $n=6$). Another oxidant, H_2O_2, also caused a detectable oxidant stress in cells and reduced LPS-induced increases in nitrite levels. Furthermore, as was the case for CSE, the inhibitory effects of H_2O_2 were prevented when cells were treated with *N*-acetylcysteine (Figure 7).

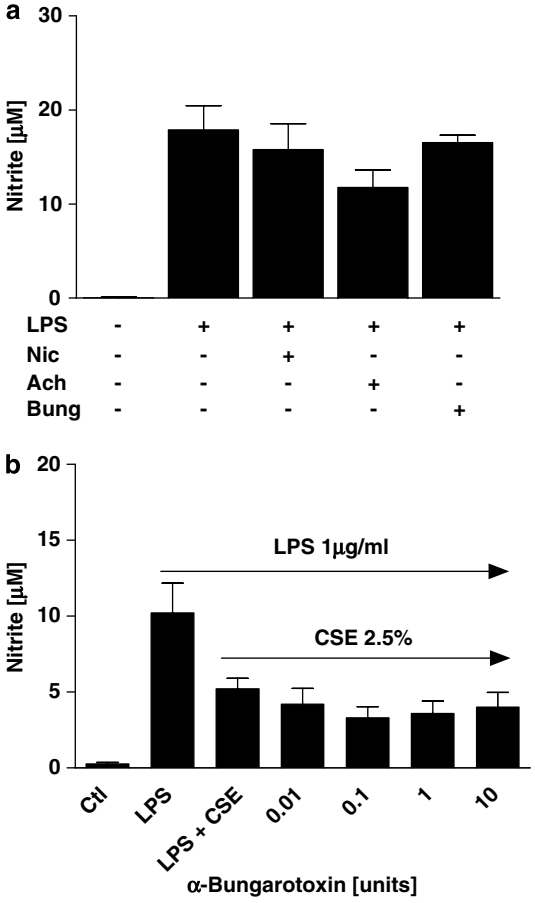

Figure 5 Effects of nicotine-related drugs on LPS (1 μg ml^{-1}) and cigarette smoke extract (CSE) induced responses in macrophages. (**a**) Cells were treated with LPS in the presence or absence of nicotine (Nic; 10 nM), ACh (Ach; 100 μM) or α-bungarotoxin (Bung; 100 U ml^{-1}) for 24 h and NO formation was measured by the levels of nitrite in culture medium. (**b**) Cells were treated with LPS (1 μg ml^{-1}) and CSE (2.5%) in the presence and absence of α-bungarotoxin (Bung, 0.01–10 U ml^{-1}) for 24 h. Data are the means ± s.e.mean; $n=3$–12 experiments. Analysis was carried out using one-way ANOVA followed by a Bonferroni post-test.

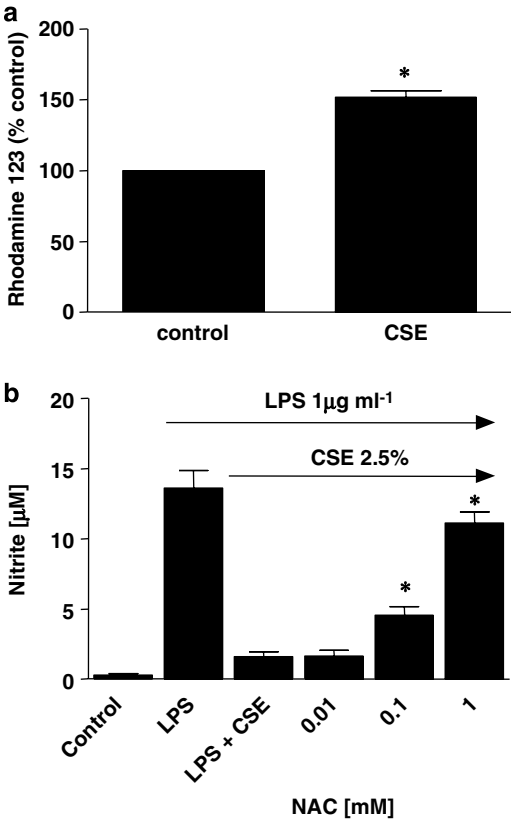

Figure 6 Role of oxidant stress in the effects of cigarette smoke extract (CSE) on NOSII activity in J774 macrophages. (**a**) Macrophages were treated with CSE for 10 min before extraction and measurement of rhodamine-123. Data are the mean ± s.e.mean; $n=3$ experiments. Data are normalized to control levels and statistical differences are measured using one-sample *t*-test for normalized data. *$P<0.05$ vs control. (**b**) Cells were treated with LPS (1 μg ml^{-1}) in the presence and absence of CSE (2.5%) with and without increasing concentrations of *N*-acetylcysteine (NAC). The data are the mean ± s.e.mean; $n=12$–21 experiments. Data were analysed by one-way ANOVA followed by a Bonferroni post-test. *$P<0.05$ vs (LPS + CSE).

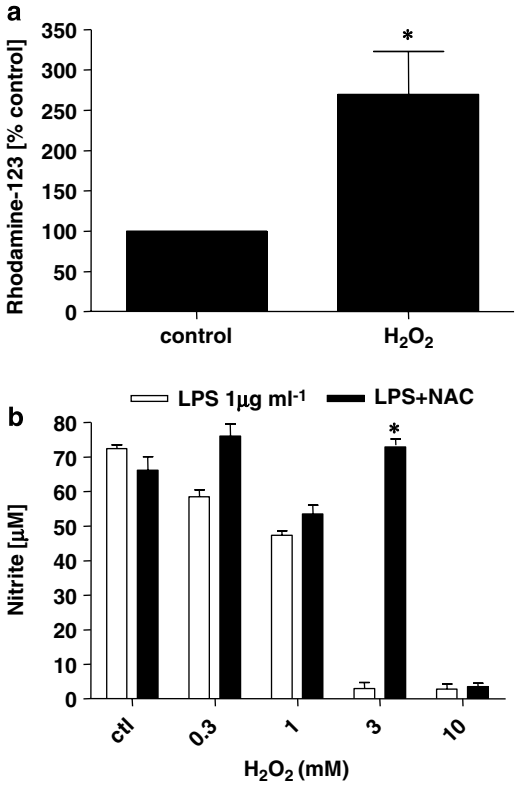

Figure 7 Effect of oxidant stress and antioxidants on LPS-induced NO release (measured as nitrite) by J774.2 macrophages. (**a**) Rhodamine-123 was measured as in Figure 6 under basal conditions and after 10 min stimulation with H_2O_2 (1 mM). Data are normalized to control levels and statistical differences are measured using one-sample *t*-test for normalized data. (**b**) J774.2 macrophages were treated with increasing concentrations of H_2O_2 (0.3–10 mM) and LPS (1 μg ml^{-1}) for 24 h in the presence or absence of *N*-acetylcysteine (NAC 1 mM). The data are the means ± s.e.mean for three experiments. Data were analysed by two-way ANOVA. *$P<0.05$ between LPS alone and LPS + NAC.

Discussion

Smoking cigarettes reduces immunity and is associated with increased risk of bacterial infection (Bartal, 2001; Laan *et al*., 2004; Sloan *et al*., 2004). In the current study, we have shown, for the first time, that CSE directly inhibits the ability of macrophages to sense whole Gram-negative bacteria at the level of NOSII induction, which is an important antibacterial mechanism (Stuehr *et al*., 1991). The inhibitory effect of CSE on whole bacterial sensing was selective because TNFα release was not reduced. The inhibitory effects of CSE were attributed to oxidants, and not to nicotine, also present in the smoke (Figure 8).

In line with previous studies within our group, the Gram-negative bacteria *E. coli* and *P. aeruginosa* caused the co-release of NO (through NOSII) and TNFα from murine macrophages (Paul-Clark *et al*., 2006). In accordance with what has been previously shown, stimulation of cells with Gram-positive bacterium *S. aureus* caused the induction of TNFα alone at this time point. In the current study, we show that a second Gram-positive bacterium *S. pneumoniae*, like *S. aureus*, induced the release of TNFα without the release of NO. Gram-negative bacteria, via LPS, activate Toll-like receptor (TLR)4 receptors (Vogel *et al*., 2003). Gram-positive bacteria, via lipoteichoic acid, activate TLR2 receptors (Vogel *et al*., 2003). TLR2 and TLR4 activation results in recruitment of MyD88 and MAL adapter proteins. TLR4 also recruits Toll/IL-1 receptor domain containing adaptor inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) adapter proteins (Vogel *et al*., 2003). TNFα is induced via the MyD88 pathway, whereas NOSII is induced via the MyD88-independent pathway (via TRIF/TRAM). Our findings, that Gram-negative bacteria induce the release of both NO and TNFα, whereas Gram-positive bacteria induce TNFα only, are therefore consistent with what is known about TLR signalling and the expression of these mediators.

In the present study, CSE inhibited the induction of NO release from murine macrophages following stimulation with Gram-negative bacteria. These observations are consistent with what is known about the effects of CSE on NOSII activity and induction *in vitro* and *in vivo* (Mazzio *et al*., 2005; Vlahos *et al*., 2006). However, ours is the first study to show a

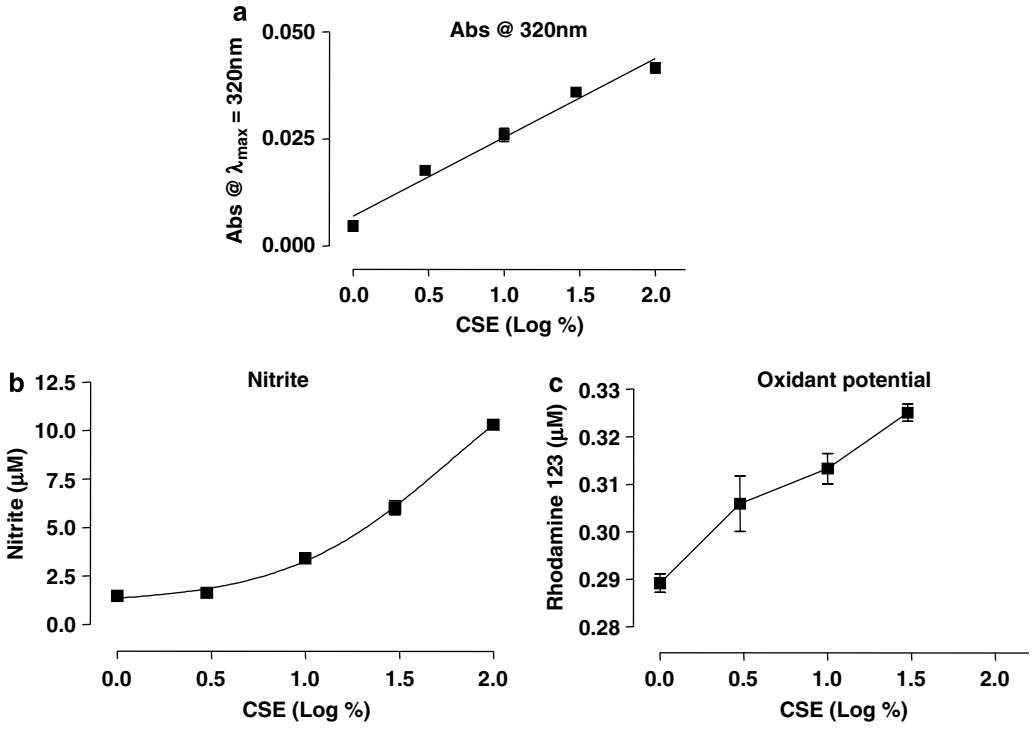

Figure 8 Comparison of units of assessment of the 'strength' of CSE. Cigarette smoke extract (CSE) was prepared in Dulbecco's modified Eagle's medium as described in the Methods section. The absorbance at 320 nm (**a**), the nitrite content (**b**) or the oxidant potential (**c**) of solutions of CSE over the range of dilutions shown were measured according to protocols described in the Methods section. The data shown are means ± s.e.mean; $n = 3$.

similar inhibitory effect of CSE on NOSII activity induced by whole bacteria and to provide a mechanism by which it acts. CSE has also been shown to inhibit the expression of other proinflammatory proteins, including interleukin-1β, interleukin-2, interferon-γ and TNFα, induced by anti-CD3 monoclonal antibody or phorbol-12-myristate-13-acetate in human monocytic cells (Ouyang *et al*., 2000), eotaxin or RANTES (regulated upon activation, normal T-cell expressed and secreted) in human airway smooth muscle cells stimulated with cytokines (Stanford *et al*., 2005) and granulocyte macrophage colony stimulation factor, and CXCL8 in human bronchial epithelial cells stimulated with LPS (Laan *et al*., 2004). Some of these proteins are important in innate immunity and inhibition of their expression by CSE affects normal immune functioning and may help to explain why smokers are more susceptible to infections (Alam *et al*., 2002).

By contrast, the expression of some proteins appear to be unaffected or induced (Walters *et al*., 2005) by CSE. In the current study, we show that in the same experiments where NOSII induction was inhibited, the release of TNFα was unaffected by CSE. The reasons why CSE regulates proteins differently in different experimental settings are not clear. However, as NOSII and TNFα are on separate TLR adapter protein pathways (Vogel *et al*., 2003), it is possible that, in our study, CSE is having an effect on the downstream TLR signalling cascade. Indeed, preliminary data from our group (Paul-Clark *et al*., 2005) and from others (Droemann *et al*., 2005; Vlahos *et al*., 2006) suggest that CSE may interact with TLR signalling. Interestingly, although CSE did not inhibit TNFα release induced by whole bacteria, it did affect the release induced by LPS. Whole bacteria will contain a range of pathogen associated molecular patterns (PAMPs), some of which interact with each other at the level of gene induction (Mitchell *et al*., 2007). It may well be that the effect of CSE, and other oxidants, on protein expression may not only be dictated by the specific gene, but also by the signal-transduction pathway leading to its expression.

How then does CSE inhibit the ability of bacteria or LPS to induce NOSII in macrophages? CSE contains a number of biologically active components, including nicotine, which may influence bacterial signalling in cells (Borovikova *et al*., 2000). These authors showed that nicotine inhibited LPS-induced activation of macrophage *in vitro* and LPS-induced 'shock' *in vivo* via an action on nicotinic receptors. The inhibitory effects of nicotine on LPS-induced responses in these models were shown to be mediated by the α7 nicotinic receptor subtype. In the current study, we found no effect of ACh (which activates muscarinic and nicotinic receptors) or nicotine (which activates nicotinic receptors) on NOSII activity in murine macrophages. Furthermore, the inhibitory effect of CSE on LPS-induced NOSII activity was unaffected by α-bungarotoxin, which is a selective nicotinic receptor antagonist, including those of the α7 nicotinic receptor subtype (Alexander *et al*., 2005). These results suggest that, in our hands, and for the NOSII pathway, nicotine does not inhibit the sensing of LPS by macrophages and is not the active inhibitory component of CSE.

CSE has previously been shown to represent a powerful oxidant stress to cells (Walters *et al*., 2005). Oxidants have

been shown to inhibit both the stability of NO (Moncada *et al*., 1986) and the induction of NOSII (Cho *et al*., 2005). To investigate the role of CSE-induced oxidant stress in its inhibitory actions on NOSII in macrophages, we pretreated cells with two well-described antioxidants, *N*-acetylcysteine and glutathione. Interestingly, both *N*-acetylcysteine and glutathione reversed the inhibition of NO release by CSE. Furthermore, the effects of CSE were mimicked by H_2O_2, which is a powerful oxidant. These observations suggest that the presence of oxidants in CSE is responsible for its inhibitory effect on NOSII in murine macrophages following bacterial stimulation. The precise mechanism by which oxidant stress downregulates NOSII induction is unclear at this time, but remains the subject of investigation. Furthermore, it is important to remember when considering the effects of CSE on different genes and in different cell types that in cells that produce high levels of NO (such as murine macrophages), CSE may have increased oxidant potential because radicals such as superoxide may combine with NO to make more powerful oxidizing species such as peroxynitrite.

In summary, we have shown that CSE can inhibit the ability of macrophages to sense and respond to Gram-negative bacteria. In our model, this inhibition is shown as a profound reduction in the ability of cells to release NO and express NOSII appropriately. However, TNFα release by bacteria was not dramatically affected by the CSE, suggesting that its effects are selective and may be mediated at the level of TLR adapter proteins. The effects of CSE appeared to be mediated by oxidant stress and not nicotine. These observations provide new insights into the mechanisms of bacteria sensing and disease.

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Conflict of interest

The authors state no conflict of interest.

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