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Effect of nicotine, cotinine and cigarette smoke extract on the neutrophil respiratory burst

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Abstract

Aims: To determine the effect of nicotine, cotinine and cigarette smoke extract (CSE) on the neutrophil respiratory burst and their effect on activation of the nuclear factor-κB (NFκB) pathway in oral epithelium.

Materials and Methods: Neutrophils from periodontally healthy individuals were treated with nicotine, cotinine and CSE before stimulation with *Fusobacterium nucleatum*, IgG-opsonized *Staphylococcus aureus* and *Escherichia coli* lipopolysaccharide. Total and extracellular reactive oxygen species (ROS) generation was determined by luminol/isoluminol chemiluminescence. Activation of NFκB in oral epithelial cells was determined by immunocytochemistry.

Results: Smoke extract alone caused increased neutrophil extracellular isoluminol-dependent chemiluminescence, not detectable with luminol. However, pre-treatment with smoke extract reduced both total and extracellular ROS generation in response to all stimuli. Nicotine and cotinine had no effect on the neutrophil respiratory burst. Smoke extract, nicotine and cotinine did not induce oral epithelial cell NFκB activation.

Conclusions: These data demonstrate that smoke extract reduces the ability of neutrophils to generate ROS after stimulation with *F. nucleatum* and IgG-opsonized *S. aureus* but, at high concentrations, stimulates extracellular ROS generation. During periodontitis, cigarette smoking may differentially affect neutrophil function, generally preventing elimination of periodontal pathogens but, in heavy smokers, also stimulating ROS release and oxidative stress mediated tissue damage.

Key words: cigarette smoke; *F. nucleatum*; neutrophil; oral epithelial cells; periodontitis; reactive oxygen species

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Periodontitis is a common, chronic inflammatory disease affecting the supporting tissues of the teeth in which cigarette smoking is the major environmental risk factor (Palmer et al. 2005). The disease involves an altered host response to specific bacteria within the subgingival plaque biofilm (Madianos et al. 2005) and a number of studies have demonstrated that peripheral blood neutrophils in periodontitis patients are both hyper-reactive and hyperactive with respect to reactive oxygen species (ROS) generation (Gustafsson & Asman 1996, Fredriksson et al. 1998, Matthews et al. 2007a). Although Fcγ-receptor (FcγR) mediated hyper-reactivity is reduced by successful non-surgical therapy, baseline, unstimulated-ROS release is not, suggesting that both reactive and constitutive mechanisms underlie the ''hyper-inflammatory'' phenotype (Matthews et al. 2007b).

Neutrophils are the major inflammatory cell at periodontitis sites and their heightened ROS generation at baseline and in response to plaque components is thought to result in collateral tissue damage and disease progression (Chapple & Matthews 2007). This is supported by the finding of reduced antioxidant defences (Brock et al. 2004, Chapple et al. 2007) together with increased levels of biomarkers of oxidative damage locally, within periodontal tissues and gingival crevicular fluid, and in the peripheral blood of

Conflict of interest and source of funding statement

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periodontitis patients (Takane et al. 2002, Panjamurthy et al. 2005). While increased levels of endogenously generated oxidants cause direct damage to periodontal tissue components (Waddington et al. 2000), it is also likely that they cause activation of redox-sensitive gene transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1, which induce pro-inflammatory cascades (Chapple & Matthews 2007).

The NADPH-oxidase complex generates the superoxide radical, following assembly and activation at the cell, lysosome or phagosome membrane surface. The latter process may be stimulated by non-physiological agonists such as phorbol 12 myristate 13 acetate (PMA), through phagocytosis and receptor independent means [direct activation of protein kinase C (PKC)], or by physiological stimulants acting via diverse receptors, such as Fcγ or toll-like receptors (TLRs). The superoxide radical then dismutates to hygrogen peroxide, a ROS, which in turn forms a number of downstream reactive species such as hypochlorous acid (via myeloperoxidase), the hydroxyl radical or peroxynitrite anion (see Chapple & Matthews 2007 for review). Luminol and isoluminol are chemiluminescent substrates, that when oxidized by hydrogen peroxide and hypochlorous acid measure real-time global ROS activity. Luminol crosses the cell membrane therefore detects both intracellular and extracellular ROS, whereas isoluminol cannot penetrate cell membranes and thus measures extracellular ROS (Dahlgren & Stendahl 1983, Dahlgren & Karlsson 1999).

Although cigarette smoking is a major risk factor for periodontitis (Palmer et al. 2005), the mechanisms underpinning the effects of smoking on periodontal inflammation remain poorly understood. In terms of neutrophil infiltration and function, smoking may have indirect effects by enhancing the pro-inflammatory activity of crevicular epithelium (via NFκB) in response to plaque bacteria (Masubuchi et al. 1998, Milward et al. 2007) and/or direct effects on neutrophils themselves. There is evidence for the former in the lung, as cigarette smoke extract (CSE) can stimulate production of neutrophil and monocyte chemotactic cytokines and activate NFκB in bronchial epithelial cells (Masubuchi et al. 1998, Liu et al. 2008). Whether oral epithelium can respond in a similar pro-inflammatory manner, and thus potentiate the response to plaque organisms, when challenged with CSE has not been reported.

Many studies have demonstrated that cigarette smoke, smoke extract or smoke condensate have direct effects on neutrophils that may contribute to periodontal tissue damage. Reported effects include increased neutrophil numbers in the peripheral circulation, increased expression of nicotinic and formyl–methionyl–leucyl–phenylalanine (fMLP) receptors, induction of protease release and impairment of chemotaxis and phagocytosis (reviewed in Palmer et al. 2005, Ryder 2007). Recently, it has also been demonstrated that CSE can induce cytokine production by neutrophils via TLRs and activation of the NFκB pathway (Mortaz et al. 2010).

In terms of ROS generation, smoking has been reported to attenuate (Sorensen et al. 2004), have no effect on (Fredriksson et al. 1999), or increase (with TNF-α priming; Gustafsson et al. 2000) the FcγR-stimulated respiratory burst of peripheral blood neutrophils isolated from smokers. By contrast, in vitro exposure of neutrophils from non-smokers to cigarette smoke or CSE has consistently demonstrated an inhibitory effect on PMA-induced ROS generation using a variety of detection methods (Ryder et al. 1998a, Zappacosta et al. 2000, Nguyen et al. 2001). However, the effect of cigarette smoke on un-stimulated neutrophils is contradictory, with stimulation (Ryder et al. 1998a) and inhibition of baseline ROS generation being reported (Corberand et al. 1980, Nguyen et al. 2001).

In line with the data on the effects of cigarette smoke/CSE, most studies have shown that nicotine inhibits ROS generation by peripheral blood neutrophils stimulated by opsonized zymosan (Sasagawa et al. 1984), fMLP and PMA (Sasagawa et al. 1985, Pabst et al. 1995), albeit at rather high concentrations (5×10^{-5} to 10^{-3} M nicotine). High concentrations of nicotine (10^{-4} M and above) have also been reported to stimulate ROS production directly (Iho et al. 2003).

To date, there have been no studies on the effect of cigarette smoke/CSE or nicotine on ROS responses of peripheral blood neutrophils to pathologically relevant stimuli, such as periodontal bacteria, bacterial endotoxin or IgG-opsonized bacteria. Furthermore, there is no consensus on the effect of smoke/CSE on baseline ROS production, the extracellular component of which is known to be elevated in neutrophils from periodontitis patients who were never-smokers (Matthews et al. 2007a).

We hypothesize that smoking (CSE) alters the level of neutrophil ROS generation in the absence of deliberate stimulation, as well as modulating TLR and/or FcγR-mediated ROS responses to endotoxin, *Fusobacterium nucleatum*, an important member of the ''orange'' complex of periodontal pathogens (Socransky et al. 1998) and IgG-opsonized bacteria. Such smoke induced changes in neutrophil ROS responses within periodontal tissues may be important in increasing oxidative stress and/or reducing innate immune responses to periodontal pathogens and their products, thereby contributing to periodontal tissue damage and disease progression. During the course of these experiments we also investigated the ability of CSE, nicotine and cotinine to activate the NFκB pathway in oral epithelium, which could indirectly increase periodontal inflammation.

Materials and Methods

Preparation of CSE

CSE in phosphate-buffered saline (PBS, pH7.4) was prepared by a method similar to those described previously (Su et al. 1998, Lee et al. 2001) using commercially available filter cigarettes (10 mg tar, 0.9 mg nicotine, 10 mg CO). A cigarette was connected to the automatic smoking apparatus, lit and the mainstream smoke drawn through the filter and bubbled through 30 ml of PBS at 37°C using suction from a peristaltic pump. The cigarettes were 100 mm long, including a 30 mm filter, and suction was stopped after 65 mm of the cigarette had been consumed over a period of 5 min. Smoke from three cigarettes was extracted into 30 ml PBS. This procedure was repeated 10 times within a 3-h period and the resultant 300 ml CSE pooled, divided into 1 ml aliquots and stored under liquid nitrogen. This stored extract had a pH of 7.2 and represented 100% CSE. Once thawed, CSE was immediately diluted in PBS for use and any unused solution discarded.

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Experimental concentrations of CSE, nicotine and cotinine

Assuming that all available nicotine was release from the 65 mm of cigarette consumed and was collected into the extract during preparation, the stored 100% stock CSE would contain a maximum of 83.6 μg/ml nicotine. As reported levels of nicotine (mean $\pm$ SE) in saliva and gingival crevicular fluid after smoking a single cigarette are 1.82 ± 0.61 μg/ml and 5.96 ± 0.77 μg/ml respectively (Ryder et al. 1998b), stock CSE was diluted (v/v) with PBS and added to cell suspensions to give final concentrations of 1%, 5% and 10%, equivalent to a maximum of 0.84, 4.2 and 8.4 μg/ml nicotine content. Similarly, experiments investigating the effects of nicotine and cotinine on neutrophil ROS generation were performed in the 0.1–10 μg/ml range, which also overlapped with systemic levels of cotinine detected in heavy smokers (median = 0.47 μg/ml; Vine et al. 1993).

Collection of venous blood and preparation of neutrophils

Periodontally healthy control volunteers ($n = 13$) were recruited from the staff of Birmingham Dental Hospital. Ethical approval was obtained from the South Birmingham Local Research Ethics Committee (Ref. no. 05/Q2707/252). All volunteers were self-reporting non-smokers (11 were never smokers) systemically healthy and exclusion criteria included a course of non-steroidal anti-inflammatory drugs or antimicrobial drugs within a 3-month period before enrolment, pregnancy or use of vitamin supplements within the previous 3 months. Venous blood was collected from the ante-cubital fossa into Vacutainer™ (Greiner, Bio-One Ltd., Stonehouse, UK) lithium heparin (17 IU/ml) tubes between 09:00 and 10:00 h. Neutrophils were isolated from blood as described previously (Matthews et al. 2007a, b) using a discontinuous Percoll gradient ($\delta = 1.079$:1.098) followed by erythrocyte lysis (0.83% NH_4Cl containing 1% $KHCO_3$, 0.04% $Na_2EDTA \cdot 2H_2O$ & 0.25% bovine serum albumen). Isolated cells were re-suspended in PBS supplemented with glucose (1 mM) and cations (1 mM $MgCl_2$, 1.5 mM $CaCl_2$; GPBS) at 1×10^6 cells/ml. Previous studies have demonstrated that both glucose and cations are required for optimal superoxide production by neutrophils (Tan et al. 1998). Although glucose alone can transiently activate neutrophils, its presence at 1mM has no effect on neutrophil metabolism and oxidant release (Kummer et al. 2007). Cell viability, typically >98%, was determined immediately before assay using dye exclusion (trypan blue).

Effect of CSE, nicotine and cotinine on H_2O_2-induced luminol and isoluminol chemiluminescence

Assays were performed without neutrophils in pre-blocked [PBS bovine serum albumen (BSA) 1%, overnight at 4°C] white microwells (Microlite2, Dynex, East Grinstead, W. Sussex, UK) as used for determination of neutrophil ROS generation. After washing the plate in PBS, GPBS was added to each well (luminol wells, 45 μl; isoluminol wells, 15 μl) followed by luminol (30 μl) or isoluminol (60 μl), horseradish peroxidase (HRP, 7.8×10^{-4} U; 20 μl, Sigma, Gillingham, Kent, UK) and diluted CSE or solutions of nicotine (Sigma) or cotinine (Sigma) (20 μl). The plate was then placed into the microplate reader (Bertold microplate luminometer LB96v) at 37°C and 60 μl pre-warmed GPBS added to each well. Light output was monitored for each well before (10 s), during and after (30 s) automatic injection of freshly prepared H_2O_2 solution (25 μl, 1%). All analyses were performed in triplicate and peak light emission, in relative light units (RLU), was determined.

CSE treatment of neutrophils

Equal volumes of diluted CSE or diluent control were added to neutrophils in GPBS (3 ml at 1×10^6/ml; 37°C) to give the required final concentrations of CSE, typically 0%, 1%, 5% and 10%. In some experiments, higher concentrations of CSE were used or nicotine and cotinine (0.1–10 μg/ml final concentration) were substituted for CSE. The tubes containing the cells were incubated at 37°C for 30 min. in a hybridization oven with the rocker set at 15 movements per minute. Neutrophils were then spun down (350 g, 6 min.), the supernatant discarded and cells re-suspended in 10 ml GPBS. After two further GPBS washes, neutrophils were re-suspended in GPBS to give a final suspension of 1×10^6 cells/ml. Preliminary experiments confirmed that this procedure effectively removed CSE from the cells, as the third wash supernatant did not inhibit H_2O_2-induced chemiluminescence (data not shown).

Neutrophil viability and caspase 3/7 activity

The viability of neutrophils treated with CSE (1–100%; 37°), nicotine and cotinine (0.1–10 μg/ml) for up to 240 min. was assessed by trypan blue dye exclusion. As neutrophils appeared refractory to CSE treatment, the effect of CSE on caspase activity was assessed to determine any pro-apoptotic effect. Caspase activity was assessed using the Promega caspase-glo® 3/7 assay kit (Southampton, UK) following the manufacturers' instructions. Cultured human oral epithelial cells (H400 cells; Prime et al. 1990, Milward et al. 2007) were used as a positive control, as pilot studies had demonstrated that they showed increased caspase activity after CSE treatment.

Lipopolysaccharide (LPS), bacterial culture and suspensions

LPS from *Escherichia coli* serotype 026:B6 was obtained from Sigma (product no. L8274). This is a short chain LPS obtained by phenol extraction from ATCC 12795. *F. nucleatum* (ATCC 10953) was grown anaerobically at 37°C as described previously (Roberts et al. 2002). Bacteria were isolated from broth cultures by centrifugation, washed three times in sterile PBS, heat treated (80°C for 10 min.) before dilution with sterile PBS to give a final stock suspension of 4×10^8 cells/ml, which was stored at −30°C. Opsonized *Staphylococcus aureus* (NCTC 6571) was prepared and stored as described previously (Matthews et al. 2007a).

Enhanced chemiluminescent assay of neutrophil ROS production

Chemiluminescence assays were performed using luminol, to detect total ROS generation (intra- and extracellular), and isoluminol, to detect extracellular ROS release. All assays were performed as described previously (Matthews et al. 2007a, b) except that neutrophils had been pre-treated with CSE, or diluent control, and washed before assay. In brief, neutrophils (1×10^5 in GPBS) were placed in pre-blocked (PBS BSA 1%, overnight at 4°C) white microwells (Microlite2, Dynex) with GPBS (30 μl), luminol (3 mmol/l; 30 μl) and PBS (15 μl) (total

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volume 75 μl) or isoluminol (3 mmol/L; 60 μl) with 6 U HRP (15 μl) (total volume 75 μl). The plate was transferred to a microplate reader (37°C, Bertold microplate-luminometer; LB96v) and baseline light output monitored for 30 min. Thereafter, cells were stimulated with opsonized *S. aureus* (NCTC 6571; MOI = 300:1; 25 μl), *F. nucleatum* (10953; MOI = 100:1; 25 μl), *E. coli* LPS (10 μg/ml; 25 μl) or PBS as control and light emission monitored for 150 min. All analyses were performed in triplicate and peak light emission, in RLU, was determined for the equilibration phase (''unstimulated'', baseline radical generation) and after stimulation.

Oral epithelial cell culture

H400 oral epithelial cells were cultured in Dulbecco's MEM (Invitrogen, Paistey, UK), containing 10% foetal calf serum (Labtech, Ringmer, E. Sussex, UK), 4 mM glutamine (Sigma), and 0.5 μg/ml hydrocortisone (Sigma), in an atmosphere of 5% CO_2 at 37°C. The cell line was derived from an oral squamous cell carcinoma, is adherent and shows a typical polygonal epithelial cell morphology with desmosomal junctions in culture (Prime et al. 1990, 1994, Milward et al. 2007). Petri dishes containing multi-well slides (CA Hendley, Loughton, Essex, UK) in growth medium were seeded with 4×10^5 cells. On day 4 (95-h culture), the cells were stimulated by addition of equal volumes of CSE, nicotine, cotinine, *E. coli* LPS or diluent (PBS) to give the required final concentrations of 10% (CSE), 50 μg/ml (nicotine and cotinine) and 20 μg/ml (*E. coli* LPS). *E. coli* LPS served as a positive control for NF-κB activation (Milward et al. 2007). After 1 or 2 h, slides were removed from the Petri dishes and washed briefly in PBS before fixation in acetone (10 min.; room temperature). Longer-term stimulation studies were also preformed as indicated in Table 2.

Immunocytochemical staining and estimation of NFκB activation

Immunostaining for NFκB and the subsequent estimation of the percentage cells demonstrating NFκB activation were performed as reported previously (Milward et al. 2007). Essentially, acetone-fixed cell monolayers were immunostained using a monoclonal antibody to p65 (clone F-6, 2 μg/ml, Santa Cruz Biotechnology, USA) and a biotin–streptavidin immunoperoxidase technique (StrAviGen, Biogenex, San Ramon, CA, USA). Counts of cells showing nuclear localization of NFκB, in the absence of cytoplasmic staining, were performed at a magnification of × 100 and the results expressed as a percentage of cells demonstrating NFκB activation.

Data handling and statistical analysis

Chemiluminescence data were recorded automatically into Microsoft Excel. Data were manipulated in Excel and statistical evaluation performed using Minitab (version 14). Kruskal–Wallis followed by Mann–Whitney *U*-test were used to determine differences between groups, using a level of $p \leqslant 0 \cdot 05$ for assigning statistical significance.

Results

Effect of CSE, nicotine and cotinine on H_2O_2-induced luminol/isoluminol chemiluminescence (cell-free system)

As it had been reported that CSE inhibits luminol chemiluminescence (Zappacosta et al. 2000), initial studies were undertaken to determine the effects of CSE, nicotine and cotinine on luminol and isoluminol chemiluminescence. CSE significantly affected both luminol and isoluminol chemiluminescence ($p < 0.0001$) with H_2O_2-induced light output being inhibited at concentrations $\geqslant 0.5\%$ ($p < 0.001$ for both substrates; Fig. 1). Both nicotine and cotinine significantly affected luminol and isoluminol chemiluminescence across the tested concentration range (0.1–50 μg/ml; $p < 0.0001$). However, nicotine had no significant effect on luminescence at low concentrations but caused significant inhibition at $\geqslant 10$ μg/ml with isoluminol and $\geqslant 20$ μg/ml with luminol ($p < 0.001$). By contrast, cotinine enhanced luminescence at concentrations ranging from 0.1–10 μg/ml with isoluminol and 0.1–5 μg/ml with luminol ($p < 0.001$; Fig. 1).

Cell viability

Cell viability was not significantly affected by CSE treatment (Table 1). However, mean viability at 50% and 100% concentrations for 2 and 4 h was lower and showed greater variation. Experiments using nicotine and cotinine at 0.1–10 μg/ml for up to 2 h similarly failed to show any significant effect of neutrophil viability.

Caspase activity

As CSE had no effect on membrane integrity as detected by dye exclusion, the possibility that it might induce apoptosis was investigated by studying caspase induction. CSE treatment for both 30 and 120 min. significantly reduced neutrophil caspase activity ($p < 0.0001$) in a dose dependent manner (Fig. 2). By contrast, CSE significantly increased caspase activity in control H400 cells ($p < 0.0001$) suggesting that it was having a pro-apoptotic effect on the oral epithelial control cells.

Effect of CSE, nicotine and cotinine on NFκB nuclear translocation in oral epithelial cells

H400 oral epithelial cells were initially treated with CSE as a positive control for the neutrophil caspase experiments. However, several additional experiments were performed to determine whether CSE, nicotine or cotinine treatment could induce nuclear translocation of NFκB. None of the treatment regimes resulted in immunocytochemically detectable nuclear translocation of NFκB (Table 2). As expected, LPS treatment of cells for 1–2 h induced high levels of NFκB nuclear translocation in H400 cells (Milward et al. 2007).

Effect of CSE on neutrophil ROS generation

Because of the inhibitory effects of CSE on both luminol and isoluminol detection systems, neutrophils were treated and then washed free of CSE before assessment of ROS generation with and without stimulation with *F. nucleatum*, opsonized *S. aureus* and *E. coli* LPS. CSE treatment had no significant effect on total, luminol-dependent pre-stimulation, baseline ROS generation ($p = 0.74$) or the chemiluminescence response to addition of PBS ($p = 0.41$; stimulation control; Fig. 3). By contrast, total ROS generation stimulated by *F. nucleatum*, opsonized *S. aureus* and *E. coli* LPS was significantly reduced by pre-treating neutrophils with CSE ($p < 0.0001$ for all stimuli). Stimulated total ROS generation was inhibited at all tested CSE concentrations except with opsonized *S. aureus*, where 1% CSE had no effect. CSE significantly affected

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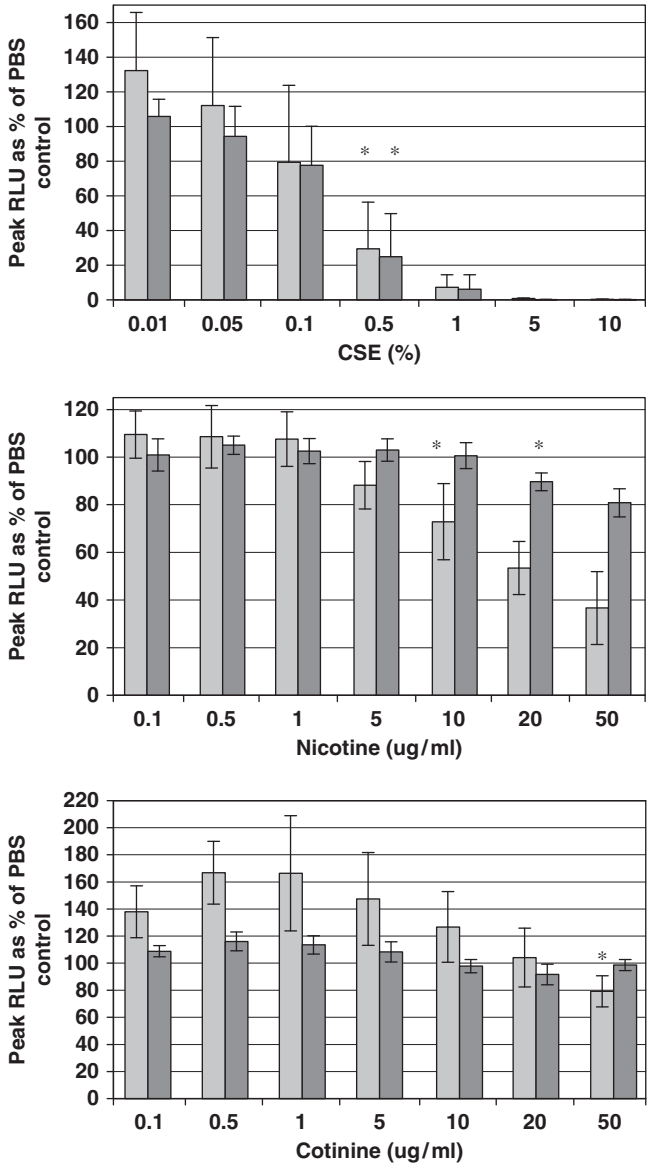

Fig. 1. The effect of cigarette smoke extract (CSE) ($n = 8$), nicotine ($n = 18$) and cotinine ($n = 18$) on H_2O_2-induced isoluminol (□) and luminol (■) chemiluminescence (mean ± SD). *Lowest concentration to give significant inhibition ($p < 0.001$). PBS, phosphate-buffered saline; RLU, relative light units.

Table 1. Effect of CSE treatment on cell viability (mean % viable cells ± SD; $n = 3$)

Treatment (min.)	% CSE					
	0	1	5	10	50	100*
30	99.4 ± 0.5	99.1 ± 0.4	98.4 ± 0.9	97.8 ± 2.5	98.6 ± 0.6	ND
60	95.3 ± 2.9	ND	ND	ND	94.0 ± 4.4	95.5 ± 3.5
120	97.5 ± 2.6	98.7 ± 0.8	98.8 ± 0.3	97.2 ± 1.9	94.4 ± 4.2	93.8 ± 4.1
240	95.8 ± 2.6	ND	ND	ND	92.8 ± 4.0	94.7 ± 3.3

*Cells spun down and pellet re-suspended in neat (100%) CSE.
CSE, cigarette smoke extract; ND, not determined.

baseline ($p = 0.004$) and control, PBS-stimulated ($p = 0.017$) isoluminol detected, extracellular ROS production, with levels being significantly increased by pre-treatment of neutrophils with 10% CSE (Fig. 3). However, extracellular ROS detected after bacterial and FcγR-stimulation was significantly affected by CSE pre-treatment ($p < 0.0001$ for all stimuli) with all concentrations resulting in reduced ROS release except at 1% with LPS stimulation.

Effect of nicotine and cotinine on neutrophil ROS generation

As nicotine had been found to have no significant effect on cell-free chemiluminescence at low concentrations (< 10 μg/ml) and that cotinine had no inhibitory effect (⩽ 50 μg/ml), neutrophil chemiluminescence was determined in the presence of these agents. Addition of nicotine and cotinine (1–10 μg/ml) did not significantly affect total or extracellular ROS generation from otherwise unstimulated neutrophils (Fig. 4). Furthermore, the chemiluminescence response to subsequent bacterial and FcγR-stimulation was also not significantly affected by the presence of either nicotine or cotinine (Fig. 5).

Discussion

Our data demonstrate that CSE pre-treatment has a profound inhibitory effect on ex-vivo neutrophil ROS generation and extracellular release primarily in response to Fcγ-receptor (FcγR; opsonized *S. aureus*) and TLR (*F. nucleatum* and *E. coli* LPS) stimulation. Thus, the continued presence of CSE during stimulation is not required to exert its inhibitory effect on both total ROS production and its extracellular release, suggesting that transient exposure during smoking a cigarette could have longer-term consequences in respect of neutrophil function within the gingival crevice/tissues. Previous reports have shown that the neutrophil ROS response to the PKC activator PMA in the presence of cigarette smoke (Ryder et al. 1998a, Nguyen et al. 2001), or after treatment with CSE, inhibits ROS generation (Zappacosta et al. 2000). However, this is the first report demonstrating down regulation of total and extracellular ROS generation after receptor stimulation using biologically and physiologically relevant stimuli and CSE concentrations (equivalent to $\sim 5 \times 10^{-6}$ to 5×10^{-5} M nicotine content).

In contrast to the above, one study suggests that addition of cigarette smoke condensate or tobacco glycoprotein before stimulation of neutrophils with fMLP increases superoxide generation by between 15% and 20% (Koethe et al. 2000). Unfortunately, data for non-fMLP-stimulated cells was not obtained,

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Table 2. Effect of CSE, nicotine and cotinine on NFκB activation in H400 cells (% cells showing nuclear staining; $n = 2$, assayed in duplicate)

Stimulation time (h)	Stimulus				
	PBS	CSE (10%)	nicotine (50 μg/ml)	cotinine (50 μg/ml)	*E. coli* LPS (20 μg/ml)
1*	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	>90
2*	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	$\geqslant 40$
72†	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	>90
96‡	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	>90
96§	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	$\leqslant 5$	>90

*Stimulus added to cells grown for 95 h.
†Medium+stimulus added 24 h after seeding, changed at 48 and 72 h; LPS added at 95 h; fixed at 96 h.
‡Cells seeded in medium+stimulus, changed at 24, 48 and 72 h; LPS added at 95 h; fixed at 96 h.
§Cells seeded in medium+stimulus and not changed; LPS added at 95 h; fixed at 96 h.
CSE, cigarette smoke extract; *E. coli*, *Escherichia coli*; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; NF-κB, nuclear factor-κB.

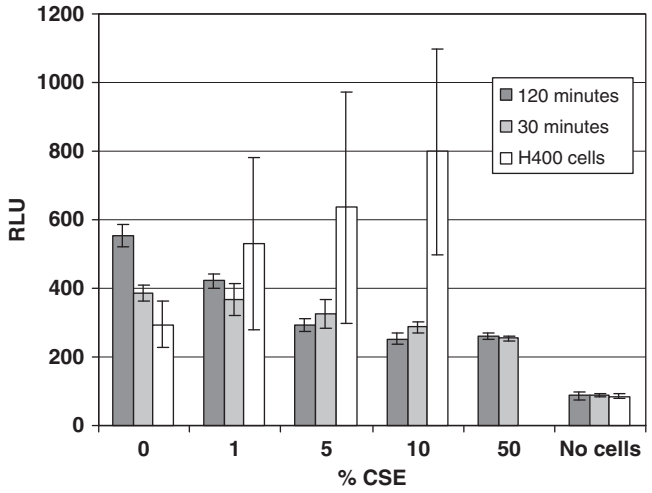

Fig. 2. Neutrophil caspase activity after 30 and 120 min. incubation with cigarette smoke extract (CSE) (1%, 5%, 10% and 50%). Caspase-glo® 3/7 reagent was then added and luminescence readings taken after 60 min. ($n = 6$). The blank (no cells) control values and data for the H400 oral epithelial cell line ($n = 10$) are shown for reference. RLU, relative light units.

so it is difficult to know whether the increased superoxide detected was due to direct stimulation by the condensate and tobacco protein. Furthermore, the condensate and tobacco protein are not equivalent to the smoke/extracts used by others.

Interestingly, our data and those of others (Ryder et al. 1998a, Zappacosta et al. 2000, Nguyen et al. 2001), agree with the findings of Sorensen et al. (2004) who demonstrated that peripheral neutrophils from ''healthy'' smokers showed reduced responses to opsonized zymosan and fMLP compared with those isolated from never smokers or smokers after 20 days abstinence. Two earlier studies investigating neutrophils from groups of periodontally healthy and diseased smokers and non-smokers failed to find a smoking-related inhibition the ROS response to FcγR stimulation (Fredriksson et al. 1999, Gustafsson et al. 2000). However, smokers in these latter studies were not on a standardized smoking regime and, on average, smoked 30% less than the well-controlled smoking group in the Sorensen study.

While the data are unequivocal in showing that CSE reduces neutrophil ROS responses to *F. nucleatum* and IgG-opsonized *S. aureus*, its direct effects on otherwise unstimulated neutrophils is less clear because CSE inhibited the chemiluminescence detection systems and had to be removed before ROS determination. The data indicate that baseline or PBS-stimulated luminol-dependent total ROS generation is unaffected by CSE pre-treatment. By contrast, there appears to be a dose-related stimulation of isoluminol-dependent extracellular ROS release at baseline and after control, PBS stimulation. Peak light output was detected very early after initiating measurements at both pre-stimulation baseline and after PBS control stimulation (data not shown) suggesting that light output was decaying from some initial high value caused by addition of CSE, when chemiluminescence could not be reliably monitored. The initial studies of Ryder et al. (1998a) demonstrated time-related stimulation of both intracellular (H_2O_2) and extracellular (superoxide) ROS production by peripheral blood neutrophils in response to exposure to gas-phase smoke. More recently, stimulation of intracellular ROS production by CSE-treated neutrophils has been reported (Mortaz et al. 2010). However, reduction of the oxidative burst of smoke-treated neutrophils in the absence of another stimulus has been reported (Corberand et al. 1980, Nguyen et al. 2001). Further studies are required, using a detection system unaffected by the presence of smoke/CSE, to clarify the situation.

These data also show that the reduced ability of CSE-treated neutrophils to generate ROS upon stimulation was not due to toxic effects of CSE. Previous studies have shown that contact with the water-soluble components of cigarette smoke generated in a smoking chamber are rapidly toxic to neutrophils (77.5% viability after 5 min.; Ryder et al. 1998a) whereas treatment with previously prepared aqueous extracts display little toxicity, even when tested within 30 min. of preparation (Dunn et al. 2005, Stringer et al. 2007, Mortaz et al. 2010). Our bulk-prepared and liquid-nitrogen stored CSE showed no toxic effects (100% CSE, 4 h) but retained the ability to significantly inhibit ROS responses to *F. nucleatum* and

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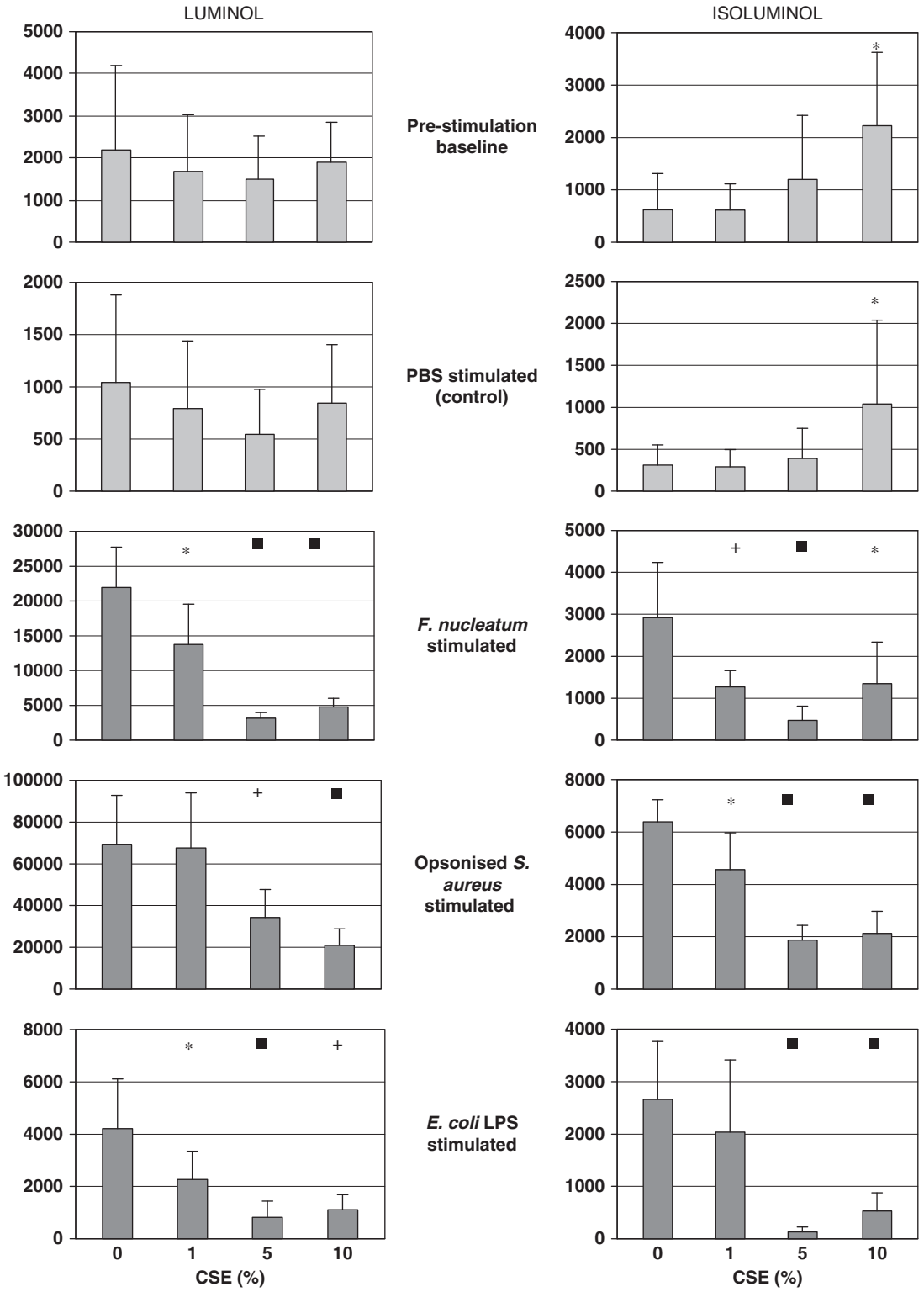

Fig. 3. The effect of cigarette smoke extract (CSE) pre-treatment on baseline and stimulated reactive oxygen species generation by neutrophils (mean peak relative light unit+SD). Comparisons with control, non-CSE-treated cells: $^{*}p \leqslant 0.01$; $^{+}p \leqslant 0.001$; $\blacksquare\ p \leqslant 0.0001$ (Mann–Whitney). LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

IgG-opsonized *S. aureus*, suggesting that the components responsible within the extract were not cytotoxic and relatively stable.

The above illustrates the potential problems with comparing and interpreting published data on the biological effects of cigarette smoke/smoke extract, namely the lack of any standardization in respect of the cigarette, presence of a filter and tobacco composition, the method of cell exposure and, if extracts are

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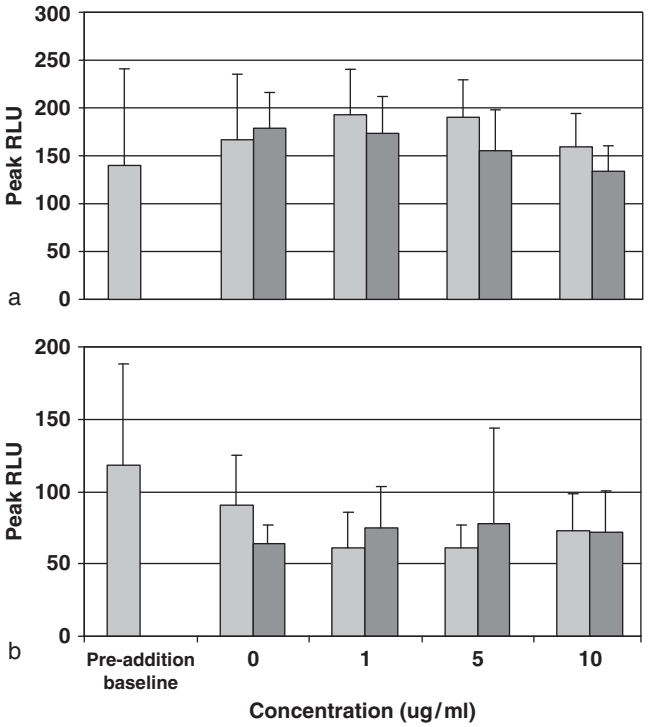

Fig. 4. Effect of addition of nicotine (■) and cotinine (■) on baseline, unstimulated peak chemiluminescence. Neutrophils were allowed to equilibrate in wells containing (a) luminol or (b) isoluminol (30 min.; pre-addition baseline) before addition of nicotine or cotinine and monitoring of light output for 30 min. (mean peak RLU+SD; $n = 6$). RLU, relative light units.

used, their preparation and the concentrations at which they are used. We have used a mainstream aqueous smoke extract at concentrations that cover those expected, in terms of potential nicotine content, to be found in saliva and gingival crevicular fluid after smoking one cigarette (Ryder et al. 1998b). Evaluation of published data is further complicated by the design of the experiments, including the medium in which ROS assays are performed and the presence/absence of glucose and divalent cations (Tan et al. 1998, Kummer et al. 2007), exposure before stimulation with/without prior removal, exposure during stimulation, length of exposure and the method of assessing ROS generation. The latter is particularly important when using luminol and isoluminol chemiluminescence. That CSE interferes with luminol chemiluminescence was first reported 10 years ago (Zappacosta et al. 2000) and has been confirmed in this study and extended to include isoluminol. However, effects of CSE on neutrophil function using both chemiluminescent substrates, in the presence of the extract, have recently been reported (Dunn et al. 2005).

Although smoke extract was not cytotoxic it did cause inhibition of caspase activity in neutrophils, agreeing with the recent data of Stringer et al. (2007) who demonstrated that CSE does not cause acute cytotoxicity or affect spontaneous neutrophil apoptosis but does directly inhibit caspase activity and impair phagocytosis. Thus, the reported reduced phagocytic activity of crevicular fluid neutrophils from periodontally healthy smokers compared with non-smokers is likely to be directly related to cigarette smoke components rather than reduced viability (Güntsch et al. 2006).

Acute CSE treatment (30–120 min.), even at the lowest dose tested, resulted in increased caspase expression by H400 oral epithelial cells indicating induction of apoptosis. This finding agrees with the only other published study of CSE exposure and apoptosis in oral keratinocytes, where acute treatment caused a robust activation of caspase 3, whereas long-term, low dose, chronic exposure (6 months) resulted in chronic caspase activation associated with protection from apoptosis (Chang et al. 2010). However, both short and longer-term (96 h) exposure of epithelial cells to CSE caused no detectable nuclear translocation of NFκB as determined by immunocytochemistry. Studies with bronchial epithelium have demonstrated both NFκB activation with associated protection from apoptosis (Liu et al. 2008) and pro-apoptotic, pirin-mediated responses to CSE (Gelbman et al. 2007) and current opinion is that, at the transcriptional level at least, oral and bronchial epithelial responses are similar (Sridhar et al. 2008, Boyle et al. 2010). The studies of Liu et al. (2008) suggest that CSE-induced inhibition of apoptosis in bronchial epithelium requires NFκB activation. The absence of any evidence that NFκB is activated in H400 cells exposed to CSE is thus consistent with the detected increased caspase activity.

Although the mechanisms by which CSE can activate NFκB have not been defined, recent research has demonstrated that CSE is able to bind TLRs (TLR 4 and 9) and induce pro-inflammatory cytokine production via NFκB activation in dentritic cells, macrophages and neutrophils (Karimi et al. 2006, Doz et al. 2008, Mortaz et al. 2009, 2010). Our preliminary data on H400 cells suggest that cigarette smoke and very high, non-physiological doses of nicotine and cotinine, in the absence of other stimuli, do not have an NFκB-mediated pro-inflammatory effect on the oral/crevicular epithelium. This is despite the fact that H400 cells show basal expression of TLR4 and nine genes which can be upregulated by stimulation with periodontal bacteria such as *P. gingivalis* and *F. nucleatum* (Milward et al. 2007). That they do not show NFκB activation in response to CSE alone may reflect a low level of membrane TLR protein expression in otherwise unstimulated epithelial cells. It would be interesting to determine the effect of CSE, nicotine and cotinine on the epithelial response to challenge with periodontal bacteria. While such a multiple challenge might be synergistic and pro-inflammatory, current concepts of the cholinergic control of inflammation via nicotinic acetylcholine receptor subunit α7 make it possible that such a combination would be anti-inflammatory (Scott & Martin 2006, Rosas-Ballina & Tracey 2009). Nicotinic acetylcholine subunit α7 receptors bind nicotine, a major component within cigarette smoke, and are expressed on keratinocytes (Grando 1997, Zia et al. 2000, Kurzen et al. 2004). Furthermore, nicotine has been shown to inhibit

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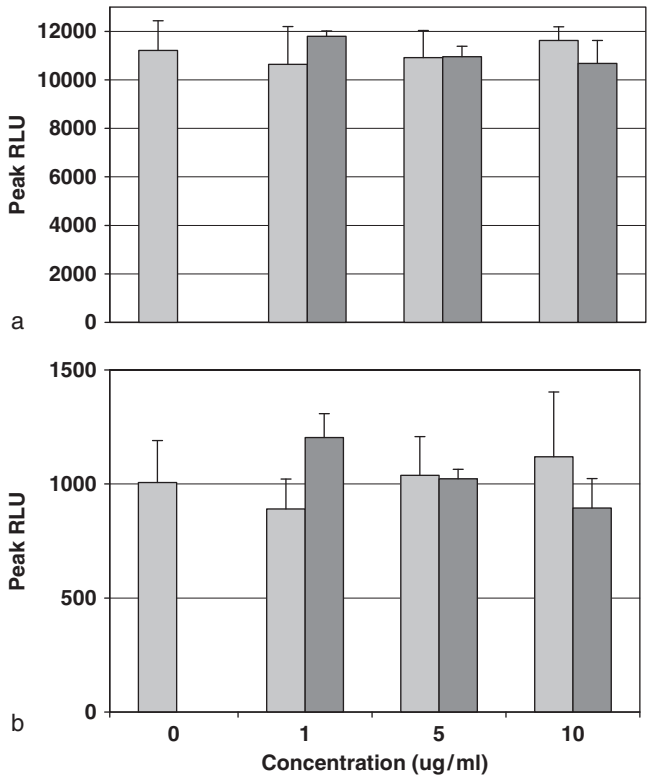

Fig. 5. Effect of nicotine (■) and cotinine (■) on *Fusobacterium nucleatum*-stimulated reactive oxygen species generation in the presence of (a) luminol and (b) isoluminol. Neutrophils were incubated with nicotine and cotinine for 30 min. before addition of *F. nucleatum* and monitoring of light output for 120 min. (mean peak relative light units + SD; $n = 6$; 0 = phosphate-buffered saline- treated control cells). RLU, relative light units.

NFκB activation in several cell types and reduce pro-inflammatory cytokine production (e.g. macrophages and endothelial cells; Scott & Martin 2006, Rosas-Ballina & Tracey 2009). In addition, knockout studies in mice have demonstrated that absence or nicotinic acetylcholine subunit α7 receptors is pro-inflammatory and associated with increased inflammatory cell infiltrates within the skin after topical application of croton oil (Gahring et al. 2010).

Unlike CSE, neither nicotine nor cotinine, at concentrations ($\sim 6 \times 10^{-7}$–6×10^{-5} M) possible in saliva, gingival crevicular fluid (Ryder et al. 1998b) and, for cotinine, plasma (Vine et al. 1993), affected baseline ROS generation or neutrophil responses to *F. nucleatum*. Thus, our data suggest that the effects on neutrophil ROS generation detected with CSE in our experimental system are not due to the presence of nicotine. These findings accord with the report of Totti et al. (1984) who similarly demonstrated that nicotine in the non-cytotoxic range (3×10^{-8}–3×10^{-4} M) had no effect on baseline or fMLP-stimulated neutrophil superoxide generation. Although there are no reports of a direct effect of nicotine on baseline ROS generation, there are several documenting nicotine-mediated inhibition of the respiratory burst generated by fMLP, PMA and opsonized zymosan (Sasagawa et al. 1984, 1985, Pabst et al. 1995) and one showing that nicotine enhances superoxide production in response to fMLP and PMA (Jay et al. 1986). These studies all use physiologically relevant nicotine concentrations except for those of Pabst et al. (1995; 6×10^{-4}–6×10^{-3} M). That the data on the effect of a single pure component of cigarette smoke are contradictory is surprising when the complex mixture of chemicals in CSE show relatively consistent inhibition of stimulated ROS generation.

A recent study has investigated the effect of physiologically relevant levels of nicotine on the DMSO-induced differentiation of HL60 cells as a model to determine whether chronic smoking would affect neutrophil maturation and differentiation (Xu et al. 2008). Nicotine contact during differentiation had no effect on subsequent baseline respiratory burst activity but inhibited, in an acetylcholine nicotinic receptor-dependent manner, the ROS-response to PMA and killing of *P. gingivalis* by differentiated cells. These data tend to support those reported showing a direct inhibitory effect of nicotine on the stimulated respiratory burst of peripheral blood neutrophils, except that nicotinic receptor dependence was not detected by Sasagawa et al. (1984, 1985). It is clear that further studies in this area are required to clarify the effects of nicotine on the neutrophil respiratory burst.

In summary our data suggest that low levels of CSE (1%; equivalent to $\sim 0.84\ \mu$g/ml, 5.2×10^{-6} M nicotine content) cause little or no effect on baseline ROS generation by neutrophils but lead to a reduced ROS response upon stimulation with *F. nucleatum* and IgG-opsonized *S. aureus*. By contrast, high doses of CSE (5–10%; equivalent to ~ 4.2–$8.4\ \mu$g/ml, 2.6–5.2×10^{-5} M nicotine content) stimulate neutrophils to generate extracellular ROS over a prolonged period (> 60 min.) as well as causing a more profound reduction in total and extracellular ROS responses upon subsequent challenge. At the level of the individual, it is interesting to speculate that light smokers would have reduced local neutrophil responsiveness to plaque organisms (reduced local innate immunity, possibly due to cholinergic control via nicotinic acetylcholine subunit α7 receptors on neutrophils; Xu et al. 2008, Gahring et al. 2010) whereas heavy smokers would have increased ''spontaneous'' generation and release of ROS by neutrophils (causing oxidative stress and periodontal damage) together with an ineffective responsiveness to plaque organisms due to reduced local innate immunity. Such a scenario could be central to smoking as a risk factor for periodontitis and explain one way in which it contributes to the development and progression of periodontitis, and the reported dose response in terms of periodontal disease severity (Martinez-Caunt et al. 1995) and tooth loss (Dietrich et al. 2007).

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Clinical Relevance

Scientific rationale for the study: Smoking is a major lifestyle risk factor for periodontitis. Mechanisms underpinning the effects of cigarette smoke on periodontal tissues remain poorly understood. Neutrophil function is strongly implicated in periodontitis pathogenesis and smoking influences neutrophil behaviour.

Principal findings: CSE reduces the ability of neutrophils to mount a respiratory burst against bacterial stimuli and these effects are not mediated by nicotine or cotinine. However, high levels smoke extract over-stimulate neutrophil oxygen radical production.

Practical implications: Smoking may reduce the ability of neutrophils to eliminate pathogens and heavy smoking may increase neutrophil-mediated collateral tissue damage.

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