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Oxygen tension modulates the cytokine response of oral epithelium to periodontal bacteria

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Abstract

Background: There is an inverse relationship between pocket depth and pocket oxygen tension with deep pockets being associated with anaerobic bacteria. However, little is known about how the host tissues respond to bacteria under differing oxygen tensions within the periodontal pocket.

Aim: To investigate the effect of different oxygen tensions upon nuclear factor-kappa B (NF-κB) activation and the inflammatory cytokine response of oral epithelial cells when exposed to nine species of oral bacteria.

Materials and Methods: H400 oral epithelial cells were equilibrated at 2%, 10% or 21% oxygen. Cells were stimulated with heat-killed oral bacteria at multiplicity of infection 10:1, *Escherichia coli* lipopolysaccharide (15 μg/ml) or vehicle control. Interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) levels were measured by enzyme-linked immunosorbent assay and NF-κB activation was measured by reporter vector or by immunohistochemical analysis.

Results: *Tannerella forsythensis*, *Porphyromonas gingivalis* and *Prevotella intermedia* elicited the greatest epithelial NF-κB activation and cytokine responses. An oxygen-tension-dependent trend in cytokine production was observed with the highest IL-8 and TNF-α production observed at 2% oxygen and lowest at 21% oxygen.

Conclusions: These data demonstrate a greater pro-inflammatory host response and cell signalling response to bacteria present in more anaerobic conditions, and hypersensitivity of epithelial cells to pro-inflammatory stimuli at 2% oxygen, which may have implications for disease pathogenesis and/or therapy.

Key words: bacteria; cytokine; epithelium; HIF-1α; NF-κB; oxygen; *P.gingivalis*

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Periodontitis is a highly prevalent, human chronic inflammatory disease. Bacteria within the dental plaque biofilm initiate the disease and progression is associated predominantly with the presence of anaerobic, Gram-negative bacteria within subgingival plaque (Farida et al. 1986). Tissue damage by reactive oxygen species and proteolytic enzymes released from the neutrophilic infiltration of the tissues are believed to be responsible for much of the host damage (Chapple & Matthews 2007, Shin et al. 2008); however, recent research has highlighted that the oral epithelial tissues also initiate a local immune reaction beyond the traditional role of physical defence (Dale 2002, Milward et al. 2007).

Pattern recognition receptors, such as the Toll-like receptors (TLR), recognize bacterial products such as lipoteichoic acid (LTA), lipopolysaccharide (LPS) and bacterial DNA. Mori et al. (2003) have demonstrated immunohistochemically TLR-2 and -4, which recognize LTA and LPS, respectively, in inflamed gingival tissues. Furthermore, TLR transcripts (TLR-2, -4 and -9) have been detected in oral epithelial cell lines (Milward et al. 2007). These TLRs are potent activators of the nuclear factor-kappaB (NF-κB) signalling pathway that ultimately induces the production of pro-inflammatory cytokines and chemokines, such as interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) (Kusumoto et al. 2004). There are many transcription factors involved in the initiation of inflammation, but

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NF-κB plays a central role. After activation, NF-κB translocates to the nucleus where it binds to κB binding sites in the promoter region of target genes, many of which code for pro-inflammatory cytokines and chemokines [e.g. TNF-α, Smith & Humphries 2009; IL-8, Hoffmann et al. 2002; IL-1β and granulocyte macrophage colony-stimulating factor (GM-CSF)]. The production of inflammatory cytokines/chemokines by crevicular epithelium in response to subgingival plaque bacteria via the TLR/NF-κB route, promoting the local influx and activation of neutrophils, is an important initial event in periodontal inflammation, as well as providing a chronic host-derived stimulus.

Studies have demonstrated that there is an inverse relationship between probing pocket depth and pocket oxygen tension (Mettraux et al. 1984, Hanioka et al. 2000). Physiologically, tissues can experience between 4% and 15% oxygen; however, the gingival tissues can also experience 21%, atmospheric oxygen conditions. At the base of untreated periodontal pockets, the oxygen tension can reach hypoxic levels at 2% oxygen (Loesche et al. 1983). This low level of oxygen is a potent immunomodulatory signal and many inflammatory sites have such low-oxygen tensions (Kivisaari 1975, Vaupel et al. 1989, Stevens et al. 1991, Bjornheden et al. 1999). Under these conditions, the hypoxia-inducible factor (HIF)-1α is the main transcription factor that is activated and is involved in adapting cell functions to cope with reduced oxygen availability (Burke et al. 2003).

Recent data have clearly shown that oral epithelial cells under normal culture conditions (21% oxygen) respond rapidly to challenge with heat-killed *Porphyromonas gingivalis* and *Fusobacterium nucleatum* to generate a pro-inflammatory response characterized by activation of NF-κB and upregulation of pro-inflammatory genes such as TNF-α, IL-1β, IL-8 and GM-CSF (Milward et al. 2007). Thus, in normoxic conditions, the subgingival biofilm has the potential to indirectly stimulate inflammation via the epithelium. Both *P. gingivalis* and *F. nucleatum* are moderately anaerobic bacteria, commonly found in deep pockets (7–10 mm; Loesche et al. 1983) with lowered oxygen tensions, which might modulate the pro-inflammatory response of epithelium to these periodontal pathogens. However, little is known about how the epithelium responds to different species of colonizing bacteria or the effects of differing oxygen tensions within the gingival crevice/periodontal pocket on such responses. Thus, this study aimed to determine epithelial NF-κB activation and pro-inflammatory cytokine production in response to nine species of oral bacteria under different oxygen tensions. The bacterial species investigated included known periodontal pathogens, mainly falling within the orange and red complexes (Socransky et al. 1998), as well as two species associated with health (*Streptococcus mitis*) or sites responding well to therapy (*Actionmyces viscosus*).

Materials and Methods

Bacteria

Bacteria were grown as described previously (Roberts et al. 2005). The bacterial strains used are listed with their ATCC classification in Table 1. Bacteria were harvested and washed with sterile distilled water and heat killed for 10 min. at 100°C. The optical density at 600 nm of each stock suspension was measured and used to calculate the approximate numbers of bacteria present (Roberts et al. 2005) for subsequent multiplicity of infection (MOI) calculations.

Cell culture

H400 oral epithelial cells were routinely cultured in DMEM/F12 supplemented with 10% foetal calf serum and glutamine under normoxic (21%) conditions. This 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, 1994a, b, Milward et al. 2007). The cell line was a gift from the originator (Professor S. S. Prime, University of Bristol, UK) but is lodged with the European Collection of Cell Cultures and available commercially from http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=06092006&collection=ecacc_gc. For experimentation, cells were plated at a density of 2×10^5/ml and allowed to adhere overnight under normoxic conditions before challenge. To obtain media at the corresponding atmospheric oxygen percentage as the experimental conditions, media was pre-equilibrated overnight in the appropriate conditions (2%, 10% or 21% oxygen; Fig. 1). This pre-equilibration regimen has been shown to eliminate fluctuations in oxygen tension seen with non-equilibrated media over a 4 h culture period (Grant & Griffiths 2007).

Winkler test for dissolved oxygen

Dissolved oxygen within culture media was measured by the Winkler test, as described previously (Grant & Griffiths 2007).

Bacterial challenge

H400 oral epithelial cells were seeded (2×10^5/ml) into white–walled 96-well plates (Corning) and allowed to adhere over night. Subsequently, media was replaced with pre-equilibrated cell culture media (2%, 10% or 21% oxygen) and cells were stimulated with bacteria (MOI 10:1): *S. mitis*, *Aggregatibacter actinomycetemcomitans*, *A. viscosus*, *Streptococcus constellatus*, *F. nucleatum nucleatum*, *Peptostreptococcus micros*, *Prevotella intermedia*, *Tannerella forsythensis*, *P. gingivalis* W83 or *Escherichia coli* LPS (15 μg/ml, Sigma, Gillingham, UK) or vehicle control. After 3 h, the media was removed from

Table 1. Classification of bacteria used to stimulate H400 oral epithelial cells

Bacteria	ATCC strain number	Socransky complex colour*
Streptococcus mitis	49456	Yellow
Aggregatibacter actinomycetemcomitans serotype *b*	43718	''Green outlier''
Actinomyces viscosus	43146	''Purple outlier''
Streptococcus constellatus	27823	Orange
Fusobacterium nucleatum subspecies *nucleatum*	25586	Orange
Peptostreptococcus micros	33270	Orange
Tannerella forsythensis	43037	Red
Porphyromonas gingivalis W83	W83	Red
Prevotella intermedia	25611	Orange

*Socransky et al. (1998).

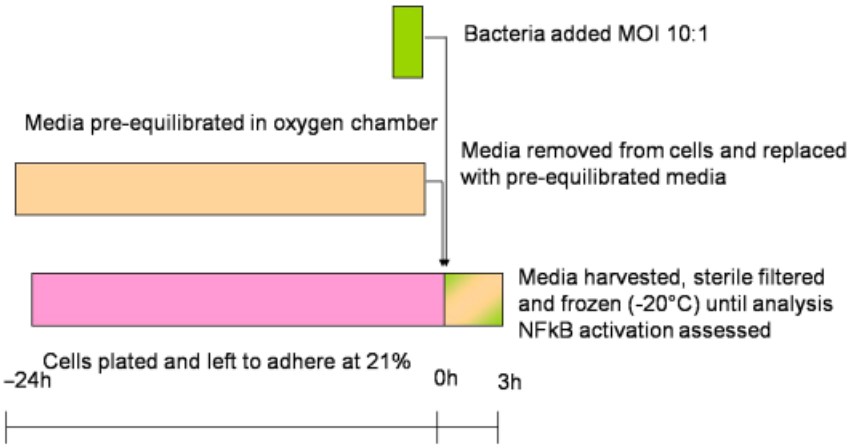

Fig. 1. Experimental procedure: H400 oral epithelial cells were plated into white-walled 96-well plates and allowed to adhere under normoxic conditions (21% oxygen). At the same time, media was put into 2%, 10% or 21% oxygen conditions to equilibrate. At the start of the experiment, media on settled cells was exchanged for oxygen-equilibrated media and the cells were stimulated with bacteria.

each well, sterile filtered and stored at -20° until assayed.

Preliminary studies examining the IL-8 and TNF-α responses of H400 cells to stimulation with *F. nucleatum* and *P. gingivalis* over a 72 h period showed that peak cytokine levels were attained after 6–8 h stimulation (data not shown). A stimulation time of 3 h was chosen because the level of cytokines obtained (IL-8; TNF-α) were well within the detection ranges of the assays and because it allowed completion of individual experiments in a manageable time frame. Similarly, preliminary experiments examining the responses of H400 cells to stimulation with *F. nucleatum* and *P. gingivalis* at different MOIs (range 10:1–300:1) showed that MOI 10:1 was the lowest ratio that elicited a response determined by IL-8 and TNF-α production and that the maximal peak was between MOI 50:1 and MOI 100:1, respectively (data not shown). Thus an MOI of 10:1 was used such that the stimulation was within a linear range for these two bacteria.

Cell viability

As most cell viability assays, including the MTT assay, are affected by oxygen tension due to the redox chemistry used for assessment of cell viability, we devised a simple assay based on the integrity of the cell membrane. After removal of media from the wells, Hoescht 33342 stain [50 μg/ml phosphate-buffered saline (PBS), Sigma] with propidium iodide (10 ng/ml PBS, Sigma) was added to the wells and incubated with cells for 30 min. before removal of the solution and addition of PBS (100 μl). Fluorescence measurements for Hoescht stain (Ho; excitation, 346 nm; emission, 460 nm) and propidium iodide (PI; excitation, 535 nm; emission, 617 nm) were used to calculate the PI/Ho ratio. As Hoescht stain can permeate into all cells, whereas propidium iodide can only enter cells where the membrane is compromised, the PI/Ho ratio is a measure of cell viability. Preliminary studies calibrating this method against the MTT assay, using H400 cells pre-treated with the cytotoxic agent hydrogen peroxide, demonstrated that a PI/Ho ratio of $\leqslant 0.01$ is equivalent to $>95\%$ cell viability (data not shown).

Cytokine measurements

Cytokine levels (TNF-α and IL-8) in cell culture media were determined by enzyme-linked immunosorbent assay (ELISA). ELISA kits were sourced from Diaclone (IDS Ltd., Boldon, UK) and the manufacturer's instructions were followed. TNF-α was detected in undiluted harvested media, whereas media were diluted 1:3 with PBS before IL-8 assay. Individual samples were analysed in duplicate and experiments were conducted in triplicate. Minimum detection limits for the analytes were: TNF-α, 25 pg/ml and IL-8, 2.5 pg/ml.

Construction of the NF-κB reporter vector

A plasmid was constructed containing 3 kB promoter sites from the 3enh vector made by Arenzana-Seisdedos et al. (1993), which was inserted into the pGL4.22 (Promega, Southampton, UK) cloning for a luciferase-tagged reporter construct with two degradation sequences, which gave lower background luminescence due to the faster removal of luciferase. Briefly, to transfer the κB promoter sites into the new vector, the restriction sites were altered. Thus, using a forward primer (5′-TTAAGCT TTTTGCAAAAGCCTAGGCC-3′) completely complimentary to the start sequence and a reverse primer (5′-AT A*GCTAGC*AGCAGCCCAGTAGTAGG-3′) containing a complimentary sequence and an overhanging sequence containing a NheI site (shown in italics), the κB promoters were amplified by polymerase chain reaction (PCR). The resulting PCR product was ligated into the pGEM T-easy cloning vector (Promega). The PCR product when inserted interrupts the promoter for a β-galactosidase gene, thus colonies of transformed JM109 competent *E. coli* (Promega) containing the PCR product were visualized using X-Gal substrate as only white colonies contained the PCR product. The resulting pGEM κB vector was then purified and the DNA of interest was isolated by digestion with HindIII (New England Biolabs, Hitchin, UK) and NheI (New England Biolabs). The new reporter vector was created by ligating the extracted κB promoter with pGL4.22 (Promega). The resulting DNA was used to transform DH5alpha competent *E. coli* and selected on ampicillin plates. Selected colonies were grown in suspension culture with ampicillin and plasmid DNA was harvested. Validation of vectors containing κB promoters was carried out by restriction digestion with HindIII and NheI, as well as by vector sequencing using GL primer 2 (Promega, data not shown).

Transfection of H400 cells

Following the successful creation of the pGL4.22 3enh vector, it was transfected into H400 cells by nucleofection, using plasmid DNA (4 μg) to transfect H400 cells (5×10^6). Transfected cells were then selected with puromycin (0.6 μg/ml). Cells were routinely kept under puromycin selection and only removed

from selection pressure during experimentation.

Detection of NF-κB induction in transfected cells

H400 oral epithelial cells containing the pGL4.22 3enh vector were seeded (2×10^5/ml) into white-walled 96-well plates and allowed to adhere over night. Subsequently, media was replaced with pre-equilibrated cell culture media (2% or 21% oxygen) and cells were stimulated with bacteria (MOI 10:1): *S. mitis*, *A. actinomycetemcomitans*, *A. viscosus*, *S. constellatus*, *F. nucleatum nucleatum*, *P. micros*, *P. intermedia*, *T. forsythensis*, *P. gingivalis* W83 or *E. coli* LPS (15 μg/ml) or vehicle control. After 3 h, the amount of luciferase produced was determined using ONE-Glo luciferase reagent (Promega). Data are expressed as a fold change over PBS treatment.

Immunohistochemical detection of NF-κB

H400 oral epithelial cells were seeded (2×10^5/ml) into 12-well plates (Greiner, Stonehouse, UK) containing 13 mm coverslips and allowed to adhere over night. Subsequently, media was replaced with pre-equilibrated cell culture media (2% or 21% oxygen) and cells were stimulated with bacteria (MOI 10:1): *S. mitis*, *A. actinomycetemcomitans*, *A. viscosus*, *S. constellatus*, *F. nucleatum nucleatum*, *P. micros*, *P. intermedia*, *T. forsythensis*, *P. gingivalis* W83, or *E. coli* LPS (15 μg/ml) or vehicle control. After 1 h, the cells attached to the coverslips were fixed with paraformaldehyde (4%, 10 min, room temperature) and permeabilized with ice-cold methanol (20 min, −20°C). Non-specific binding was blocked by washing with 1% bovine serum albumin (BSA) in PBS and then p65 was located with Alexa-fluor 288-conjugated anti-p65 antibody (dilution 1:50, sc8008-AF488, Santa Cruz, Heidelberg, Germany). Nuclei were counterstained with Hoescht stain (10 ng/ml). Coverslips were then mounted in MOWIOL (Calbiochem, Darmstadt, Germany) containing an anti-fade compound. Immunostained cells were visualized using a Leica DMRB microscope equipped with a Hamamatsu ORCA camera, and images were captured and processed using OpenLab software (Improvision, Perkin Elmer, Cambridge, UK) and processed with ImageJ and Abode Photoshop 6.0.

HIF-1α knockdown

H400 oral epithelial cells were seeded (1×10^5/ml) in 12-well plates or H400 oral epithelial cells containing the pGL4.22 3enh vector were seeded in (1×10^5/ml) 96-well white-walled plates. Cells were then transfected with 12 pmol small interfering RNA (siRNA) or 1 pmol siRNA, respectively, using lipofectamine RNAiMAX (Invitrogen, Paisley, UK) for 48 h. siRNA was sourced from Qiagen (Crawley, UK) (Hs_HIF1A_6 sequence AGGAAGAA CTATGAACATAAA). Control wells were transfected with non-silencing control (Qiagen All Stars Negative Control siRNA) in the same way. After 48 h, media was removed and replaced with media pre-equilibrated at 2% or 21% oxygen and the cells were stimulated with bacteria (MOI 10:1). Media from 12-well plates was harvested after 3 h and luciferase was determined in 96-well plates after 3 h, using ONE-Glo luciferase reagent (Promega).

Western blotting

HIF-1α knockdown was confirmed using Western blotting. H400 cells were harvested after 24 h treatment with siRNA into Laemlli buffer (Sigma). After boiling, proteins were separated by SDS-PAGE and electroblotted onto a PVDF membrane. The membrane was blocked with BSA (3% in PBS) for 1 h before incubation with anti-HIF-1α (dilution 1:50, sc-10790, 16 h, 4°C, Santa Cruz). After washing, the membrane was incubated with goat anti-rabbit HRP-conjugated secondary antibody. Proteins were visualized with the chemiluminescence substrate SuperSignal West *Pico ECL* Substrate (Pierce, Cramlington, UK).

Statistical analysis

All data were analysed using two way ANOVA, with Graph Pad Prism v 3.03.

Results

Incubation of media in 2%, 10% or 21% oxygen atmosphere over 24 h resulted in media with dissolved oxygen concentrations of 3.0 ± 0.5, 3.5 ± 0.5 and 4.0 ± 0.5 mg/ml at 37°C, as determined by the Winkler test. Cell viability after culture in these differing oxygen conditions and following treatment with the nine species of oral bacteria (MOI 10:1), LPS (15 μg/ml) or vehicle control showed some variation (Fig. 2a). However, the viability of all bacterially and LPS challenged cultures was >95% (PI/Ho ratio <0.01) and there were no significant differences due to either oxygen tension or bacterial stimulation (two way ANOVA $p = 0.2315$ and 0.1680, respectively) over the course of the experiment.

Media from H400 cells grown under differing oxygen conditions and treated with the nine species of oral bacteria (MOI 10:1), LPS (15 μg/ml) or vehicle control were assessed for IL-8 and TNF-α (Fig. 2b and c). Although large amounts of IL-8 were produced by unstimulated cells (mean ± SEM: 1003 ± 489 pg/ml), production was generally increased when the cells were stimulated with bacteria or LPS. There was a trend among all bacterial treatments for greater quantities of IL-8 to be produced at 2% oxygen tension over 10%, which was in turn >21% (Fig. 2b). While two way ANOVA analysis showed that this trend was not statistically significant ($p = 0.3117$), there were significant effects due to the species of bacteria used to challenge the epithelial cells ($p = 0.0047$). The nine species of bacteria could be subdivided into three groups according to their ability to induce IL-8 production. *T. forsythensis*, *P. gingivalis* W83 and *P. intermedia* all induced high levels of IL-8 (mean ± SEM 3031 ± 471 pg/ml, range = 2020–4130 pg/ml) that were similar to those obtained with *E. coli* LPS and consistently three- to fourfold greater than that produced by PBS-treated controls (mean ± SEM 1167 ± 191 pg/ml; range = 440–1598 pg/ml; $p = 0.0380$). Similarly, *A. actinomycetemcomitans* and *F. nucleatum nucleatum* induced IL-8 levels greater than PBS-treated controls, albeit at a lower mean range (mean ± SEM 1853 ± 408 pg/ml; range = 300–5694 pg/ml; $p = 0.1711$) than found for *T. forsythensis*, *P. gingivalis* W83, *P. intermedia* and *E. coli* LPS. The remaining bacteria tested had little effect on IL-8 production by H400 cells under the conditions used.

TNF-α, produced in small amounts by unstimulated cells (mean ± SEM 21.7 ± 9.3 pg/ml; range = 0–71.1 pg/ml), was induced by *P. intermedia*, *T. forsythensis P. gingivalis* W83 and *E. coli* LPS, with *P. intermedia* showing the largest and most consistent effect (Fig. 2c). *P. intermedia* also showed the trend for greater cytokine production at 2% oxygen tension over 10%, which

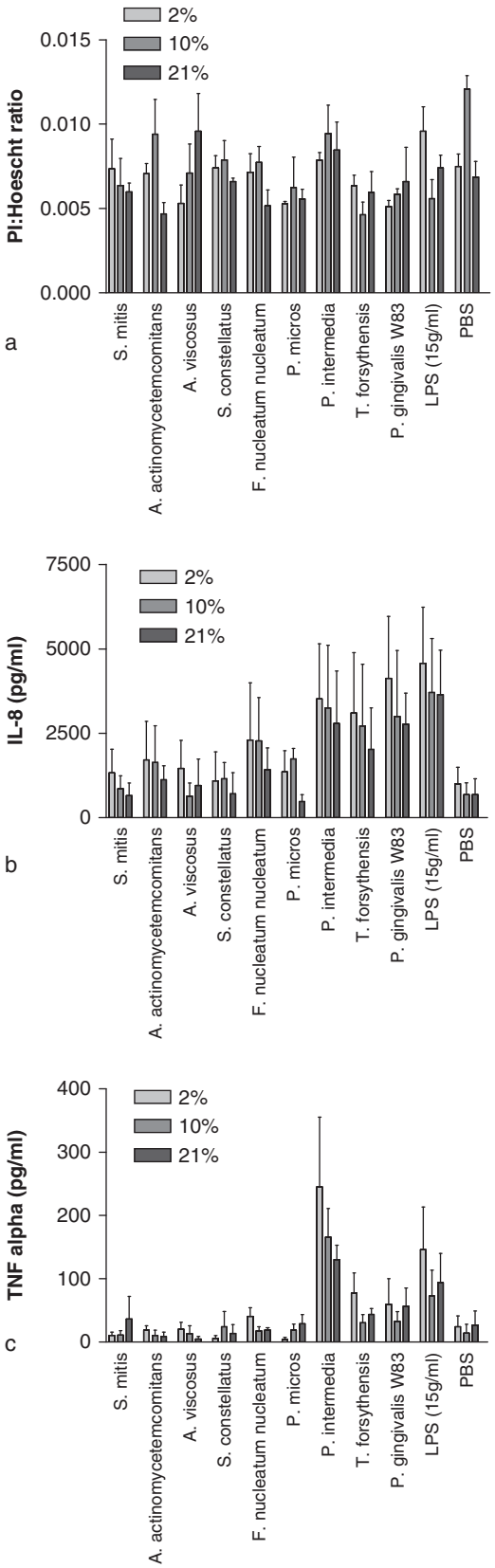

Fig. 2. Effect of bacterial stimuli on H400 cells under a range of oxygen conditions. (a) Cell viability was assessed using propidium iodide exclusion in ratio to Hoescht uptake for total cells ($n = 3$). (b) Interleukin-8 (IL-8) and (c) tumour necrosis factor-alpha (TNF-α) levels detected in media after cells were stimulated with bacteria, under different oxygen conditions ($n = 3$ separate experiments, individual experiments were measured in duplicate).

was >21%. Statistical analysis of the whole dataset revealed that the bacteria had an effect on the production of TNF-α ($p < 0.0001$), and this was not significantly modulated by oxygen tension ($p = 0.2162$).

Immunohistochemical analysis of NF-κB translocation indicated that this was affected by both bacterial stimulation ($p < 0.0001$) and oxygen tension ($p < 0.0001$) (Fig. 3a and b). In particular, *T. forsythensis, P. gingivalis* W83 and *P. intermedia* stimulated NF-κB translocation to a similar degree as *E. coli* LPS and more than any of the other bacterial species examined. The NF-κB reporter vector showed that while bacteria had a significant effect on NF-κB activation of luciferase expression ($p = 0.0119$), oxygen tension had no effect ($p = 0.9021$, Fig. 3c). Once again, *T. forsythensis*, *P. gingivalis* W83 and *P. intermedia* stimulated H400 cells above the levels induced by the remaining bacteria and seen in PBS-treated controls.

As there was a suggestion from the previous data that levels of cytokines secreted and NF-κB activation might be modulated by lower oxygen conditions, preliminary studies were performed to investigate the involvement of HIF-1α using siRNA to knock down HIF-1α protein expression. HIF-1α siRNA treatment of H400 oral epithelial cells resulted in an approximate 30% reduction in cellular HIF-1α protein determined by scanning densitometry of Western blots (Fig. 4a and b). Choosing the most effective bacterial modulators of NF-κB activation, IL-8 and TNF-α production (*T. forsythensis*, *P. gingivalis* W83, *P. intermedia*), the effect of decreased HIF-1α was examined. The NF-κB reporter assay demonstrated that HIF-1α siRNA reduced the activation of the NF-κB response (Fig. 4c). IL-8 secretion was not altered in response to the decreased HIF-1α expression at 2% and 21% oxygen tensions ($p = 0.9926$ and 0.9214, respectively; Fig. 4d). By contrast, the TNF-α response of epithelial cells treated with HIF-1α siRNA to *T. forsythensis*, *P. gingivalis* W83, *P. intermedia* or LPS (15 μg/ml) was decreased at 2% and 21% oxygen tensions ($p < 0.0001$ for both oxygen tensions, Fig. 4e).

Discussion

The epithelium functions as the first line of defence against oral bacteria, provid-

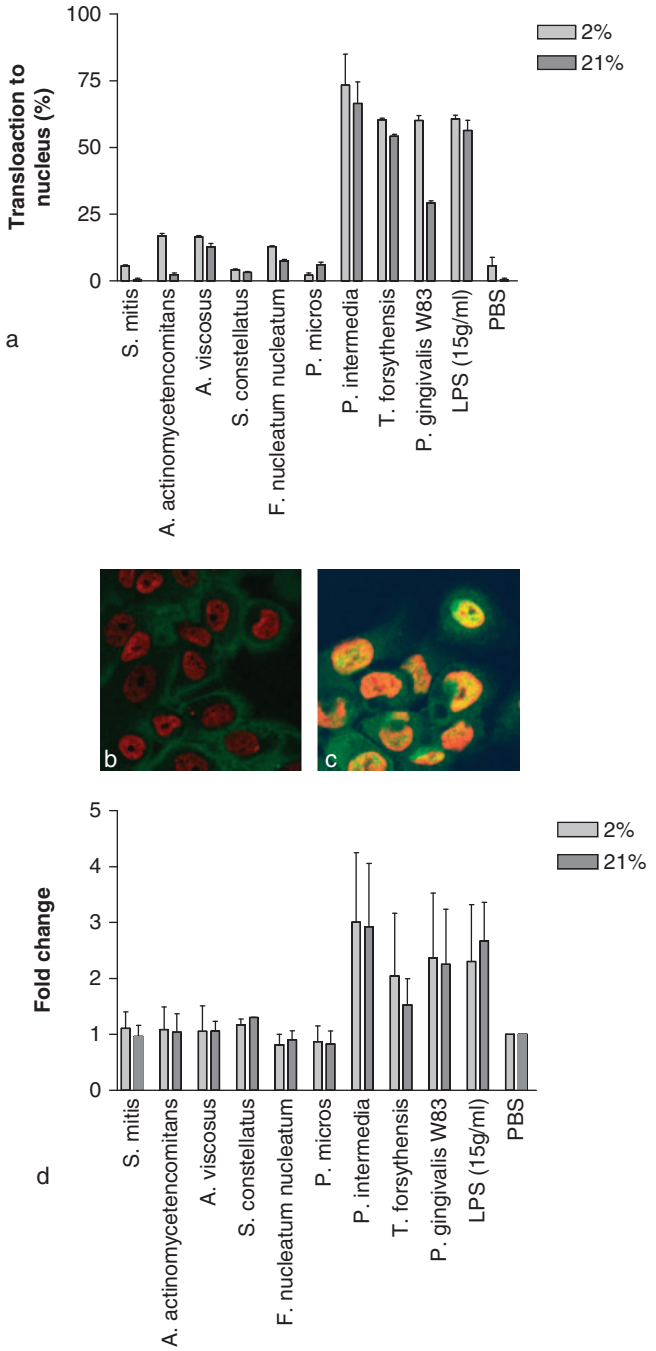

Fig. 3. Effect of bacterial stimuli on nuclear factor-kappaB (NF-κB) activation in H400 cells under a range of oxygen conditions. (a) Percentage NF-κB nuclear translocation detected in cells stimulated by bacteria, under different oxygen conditions ($n = 3$). Example of immunohistochemical staining of H400 cells, p65 (green), Hoescht DNA stain (falsely coloured red): (b) phosphate-buffer saline (PBS) stimulated cells; (c) lipopolysaccharide (LPS) stimulated cells. (d) NF-κB activation in cells stimulated by bacteria, under different oxygen conditions, using the NF-κB reporter vector ($n = 3$).

ing both a physical and responsive barrier. The response of the epithelium is to produce antimicrobial factors (e.g. human β-defensin-2) and to alert the innate and adaptive immune systems through the production of pro-inflammatory and immunomodulatory molecules (e.g. IL-1α and IL-1β, IL-8 and TNF-α), after activation of transcription factors such as NF-κB (Krisanaprakornkit et al. 2000, Milward et al. 2007, Yin & Dale 2007). In this study, we have investigated the effect of a range of oral bacteria, including examples of both red and orange complex organisms (Socransky et al. 1998), on the cytokine and NF-κB response of an oral epithelial cell line and how this is modulated by oxygen tension. These data demonstrated that oral epithelial cells consistently show a greater response to *T. forsythensis*, *P. gingivalis* W83 and *P. intermedia* as measured by nuclear translocation and activation of NF-κB and production of IL-8 and TNF-α.

Thus, two red complex bacteria, *T. forsythensis* and *P. gingivalis* W83, and the orange complex bacterium *P. intermedia* induced the greatest pro-inflammatory epithelial response at all oxygen concentrations tested. It has long been considered that approximately 80% of the variance in periodontal disease is due to host responses (Grossi et al. 1994) and these bacteria, known to be associated with the deepest pockets in vivo (Socransky et al. 1998), have the capacity to indirectly promote periodontal inflammation via the epithelium and contribute to connective tissue destruction and increased pocket depth, even under lower oxygen conditions. This ability (virulence factor) is either not shared by the other bacteria tested or is only expressed to a lesser degree. For example, *F. nucleatum* and *A. actinomycetemcomitans serotype b* induce epithelial production of IL-8 and nuclear translocation of NF-κB, particularly at low oxygen levels. Thus, while *P. intermedia* is able to induce significant pro-inflammatory epithelial cell responses, other tested members of the orange complex, do not, suggesting that their pathogenic potential may be independent of epithelial stimulation. Interestingly, periodontal bacteria function within complex biofilms and certain ''clusters'' are associated with more severe periodontitis than others (Socransky et al. 1998). The most pathogenic complex (red complex) comprises *P. gingivalis, T. forsythensis* and *Treponema denticola* (not tested in these studies) and taken together, these three organisms alongside *P. intermedia* induced a substantially greater cytokine response, irrespective of oxygen tension.

An oral epithelial cell line was chosen over using primary cells to overcome the difficulties with reproducibility when using primary cultures, particularly in light of the number of bacteria and culture conditions tested. In addition, use of the cell line enabled the NF-

κB reporter studies and the HIF-1α knockdown experiments to be performed. Previous work has demonstrated that H400 cells are similar to primary oral epithelial cells with respect to epithelial growth factor (EGF) and transforming growth factor (TGF)-β receptor expression, response to exogenous EGF and TGF-β, as well as autocrine production of TNF-α (Prime et al. 1994a, b). H400 cells have also been shown to respond to heat-inactivated *F. nucleatum* and *P. gingivalis* via NF-κB activation resulting in an upregulated expression of pro-inflammatory cytokine genes (e.g. IL-1β and IL-8; Milward et al. 2007). Furthermore, the original observations that junctional epithelium in inflamed periodontal tissues exhibit IL-8 gene expression (Tonetti et al. 1994) and that pro-inflammatory gene expression is upregulated in primary oral epithelial cells in response to infection with *P. gingivalis* (Sandros et al. 2000), support the validity of using H400 cells in our model test system.

In a recent paper by Stathopoulou et al. (2010), primary cultures of gingival epithelial cells were challenged, under normoxic conditions, with either live or heat-killed *P. gingivalis* (ATCC 33277), *F. nucleatum* 364 or *A. actinomycetemcomitans* Y4 at an MOI of 10:1 (directly comparable to the data presented here) and 100:1. *F. nucleatum* (live and heat-killed) was the only bacterium to consistently induce epithelial IL-8 production. By contrast, epithelial IL-8 was only stimulated at high MOI by heat-killed *P. gingivalis* and live *A. actinomycetemcomitans*. The inability to detect IL-8 in culture supernatants

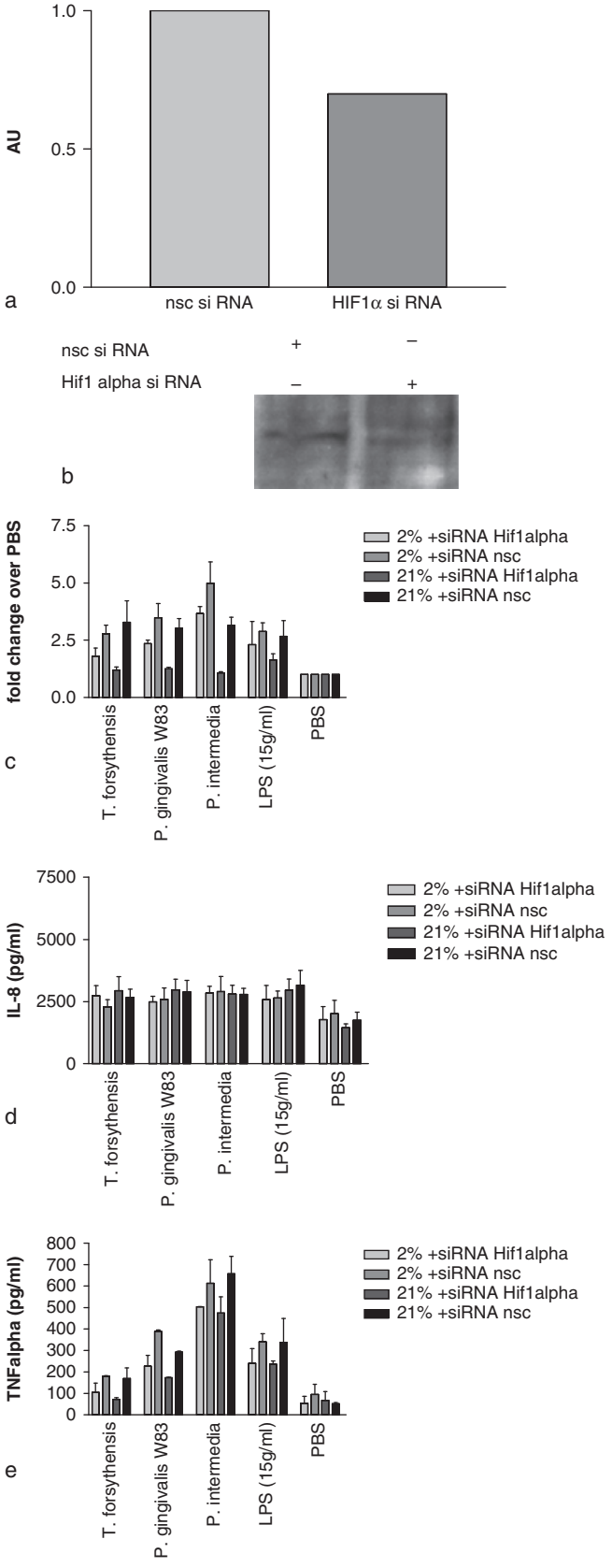

Fig. 4. Effect of hypoxia-inducible factor (HIF-1α) small interfering RNA (siRNA) on H400 oral epithelial cell nuclear factor-kappaB (NF-κB) activation and cytokine production by bacterial stimuli under different oxygen conditions. (a) Quantitation of Western blot by scanning densitometry (AU: arbitrary units) showing reduction in HIF-1α protein levels after 48 h with HIF-1α siRNA or non-silencing control (nsc) siRNA before and then 3 h at 2% oxygen. (b) Western blot showing reduction in HIF-1α protein levels after 48 h with HIF-1α siRNA or nsc siRNA and then 3 h at 2% oxygen. (c) Cells were treated with HIF-1α siRNA or nsc siRNA and NF-κB activation was measured in cells stimulated by bacteria, under different oxygen conditions, using the NF-κB reporter vector ($n = 3$). (d and e) Cells were treated with HIF-1α siRNA or nsc siRNA, stimulated with bacteria under different oxygen conditions and levels of IL-8 or TNF-α in media determined by enzyme-linked immunosorbent assay (ELISA) ($n = 3$ separate experiments, individual experiments were measured in duplicate).

of oral epithelial cells mono-infected with high numbers of live *P. gingivalis*, even though the gene is expressed, has been known for a long time (Madianos et al. 1997, Darveau et al. 1998) and thought to be the result of degradation of secreted IL-8 by *P. gingivalis*-derived proteases, particularly gingipains (Darveau et al. 1998, Mikolajczyk-Pawlinska et al. 1998, Zhang et al. 1999). While such degradation might lead to reduced local inflammation, release of more biologically active, lower molecular weight forms of IL-8 have been reported, both in terms of chemo-attraction of neutrophils and priming for the neutrophil respiratory burst (Mikolajczyk-Pawlinska et al. 1998, Dias et al. 2008).

Our data for *F. nucleatum* and *P. gingivalis*, using low-dose challenge of a carcinoma-derived oral epithelial cell line with non-viable bacteria, broadly agree with the recent study of Stathopoulou et al. (2010) and extend it to demonstrate that similar processes are possible in the low-oxygen conditions present in untreated periodontal pockets (Loesche et al. 1983, Mettraux et al. 1984). The apparent greater induction of epithelial IL-8 by *P. gingivalis* in our study may reflect the fact that we used *P. gingivalis* W83 whereas Stathopoulou et al. (2010) used *P. gingivalis* ATCC 33277. The latter strain is known to be less virulent, in terms of inducing subcutaneous abscess formation in animal models (Grenier & Mayrand 1987, Sundqvist et al. 1991, Laine & van Winkelhoff 1998), and more recently, it has been shown that *P. gingivalis* ATCC 33277 lacks 10 genes expressed by *P. gingivalis* W83 that associate with periodontitis (Lin et al. 2009).

Our data demonstrate that NF-κB translocation to the nucleus was the only marker significantly affected by oxygen tension but that similar, non-significant trends were seen for NF-κB activation and epithelial production of IL8 and TNF-α. However, previous studies have demonstrated that hypoxia causes oxidative stress and both nuclear translocation and activation of NF-κB (Koong et al. 1994, Sarada et al. 2008). Receptor activation results in the redox-regulated phosphorylation and the subsequent destruction of I-κB allowing early and rapid translocation of NF-κB from the cytoplasm to the nucleus. This initial event is likely to be affected most by oxygen tension, rather than the later events (activation of the reporter and cytokine production) where the effect of oxygen tension is diluted by subsequent biochemical interactions.

HIF-1α is an integral part of the NF-κB response, such that reductions in NF-κB prevent HIF-1α gene expression. However, levels of HIF-1α protein are dependent upon oxygen tension and the protein is rapidly degraded under normoxic conditions (Jung et al. 2003, van Uden et al. 2008). Although HIF-1α is the main transcription factor activated under low-oxygen conditions, it is, in concert with NF-κB, also a key transcription factor activated by cytokines, reactive oxygen species and bacterial LPS under normoxic conditions (Jung et al. 2003, Déry et al. 2005, Frede et al. 2006). In our study using HIF-1α siRNA, reducing the amount of HIF-1α protein in bacterially challenged epithelial cells caused reductions in TNF-α production in both normoxic and reduced (2%) oxygen conditions. By contrast, the IL-8 response was not affected. This is consistent with the fact that the IL-8 gene does not have a promoter sequence for HIF-1α (HRE) (Shi et al. 1999), whereas tumour necrosis factor-α converting enzyme (TACE) is regulated by HIF-1α (Charbonneau et al. 2007). Therefore, it appears that epithelial TNF-α production in response to *T. forsythensis*, *P. gingivalis*, *P. intermedia* and *E. coli* LPS is partially dependent upon HIF-1α activation under both low oxygen and normoxic conditions. A surprising finding was that a partial inhibition of HIF-1α associated with a decrease in NF-κB reporter activation under both 2% oxygen and normoxic conditions. This may highlight a bi-directionality in the relationship between these two transcription factors and reflect the complexity of their interaction. However, further siRNA studies are required to confirm these preliminary observations on the role of HIF-1α in the pro-inflammatory response of oral epithelial cells to periodontal bacteria.

Data presented here show that *T. forsythensis*, *P. gingivalis* and *P. intermedia* induce high levels of epithelial IL-8 and TNF-α expression, and thus it may be expected that increased concentrations of these cytokines should be found in gingival crevicular fluid (GCF) collected from diseased sites containing these bacteria. Published reports on cytokine levels in GCF are complex. There are several cross-sectional comparisons demonstrating raised concentrations of GCF IL-8 in patients with moderate to severe periodontitis compared with health (Gamonal et al. 2001, Offenbacher et al. 2007) and others showing the opposite (Chung et al. 1997, Jin et al. 2002). However, the three studies in which IL-8 levels were analysed longitudinally, pre- and post-treatment, consistently showed a mean reduction in IL-8 concentrations in all (Gamonal et al. 2001, Jin et al. 2002) or a majority of the patients investigated (Chung et al. 1997). This difference between studies may reflect the short time period between therapy and GCF collection in the latter case (2 weeks) compared with the 1–2 months in the other studies. A confounding factor in the interpretation of IL-8 levels in GCF is the fact, discussed previously, that the accumulation of epithelial IL-8 in response to *P. gingivalis* depends upon their viability (infectivity). Thus, the proportion of viable to non-viable *P. gingivalis* within subgingival plaque could alter local levels detectable in GCF. Furthermore, *P. gingivalis*, at certain levels of infection, is also known to down-regulate epithelial IL-8 production in response to other bacteria, including *F. nucleatum* (Madianos et al. 1997, Darveau et al. 1998). Although not completely understood, this activity is associated with the production of the phosphoserine phosphatase SerB by *P. gingivalis*, which is thought to modulate epithelial cell signal transduction (Hasegawa et al. 2008). Thus, local host cell IL-8 production in response to various components of the subgingival biofilm may be significantly modulated by the number of viable *P. gingivalis* present.

The picture for TNF-α is also complex as levels often fall below the limit of detection in health. However, low levels have been detected in GCF from periodontitis patients (3.2 pg/ml, Yavuzyilmaz et al. 1995; 0.51 pg/ul, Erdemir et al. 2004). Detectable amounts of TACE have also been measured in periodontitis, and found not to be elevated in comparison with health, whereas they are in comparison with gingivitis (Bostanci et al. 2008) with an overall positive correlation with pocket depth, perhaps indicating a relationship with HIF-1α and oxygen-associated pocket depth decreases.

In the hypoxic lung, there are increases in cytokines including IL-8 that correlate with inflammation and epithelial cell injury (Bhandari & Elias 2006). Similarly, reductions in oxygen tension in the lung enhance Gram-negative sepsis (Matuschak et al. 1998), and

a hypoxic environment also enhances LPS-induced TNF-α and IL-1β in alveolar macrophages, which bind opsonized particles more efficiently than under normoxic conditions (Acosta-Iborra et al. 2009). However, neutrophils under hypoxic conditions produce less IL-8, TNF-α and IL-1β than under normoxic conditions, when stimulated by LPS (Derevianko et al. 1996), but how they react to cytokine stimulation under these conditions is not known. This is of potential importance as our data clearly show that pocket epithelium has the capacity to produce IL-8 in response to periodontal pathogens and to chemoattract peripheral blood neutrophils, the inflammatory cells are considered the most important in periodontitis and thought to play a central role in host-mediated damage via the release of lysozomal enzymes and generation of reactive oxygen species (Buchmann et al. 2002, Chapple & Matthews 2007). Although periodontitis is associated with the presence of hyperactive peripheral neutrophils in terms of ROS generation in response to bacterial stimulation (Matthews et al. 2007a, b) it remains to be proven if they retain this ability under the low-oxygen conditions within the periodontal pocket.

Few studies have looked at physiological changes in oxygen tension in the periodontal pocket (Loesche et al. 1983, Mettraux et al. 1984, Hanioka et al. 2000) and little is known about how hypoxia affects the currently accepted mechanisms underlying the pathogenesis of periodontitis. Here, for the first time, we have examined how oxygen levels impact upon inflammation and the epithelial pro-inflammatory response to a range of oral bacteria. Our data demonstrate that pro-inflammatory responses of epithelium to subgingival plaque organisms are maintained and potentially increased under the oxygen levels detected in vivo. The data also demonstrate that the pathogenic potential of *T. forsythensis*, *P. gingivalis* W83 and *P. intermedia* is reflected by their ability to induce pro-inflammatory epithelial responses and illustrate the need for further work to increase our understanding of microbial–epithelial cell interactions in periodontal disease

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Clinical Relevance

Scientific rationale for study: Epithelial surfaces generate molecular signals in response to colonizing bacteria, which initiate inflammatory responses in the connective tissues. Periodontal studies have to date been performed under atmospheric oxygen tensions, yet the pocket environment becomes progressively more anaerobic towards its base.

Principal findings: Here we show that ''red complex'' bacteria are the most potent stimulators of oral epithelial cell cytokine release and that such responses appear enhanced at low-oxygen tensions.

Practical implications: Contrary to traditional theory, the anaerobic pocket environment maintains and may enhance epithelial pro-inflammatory responses to pathogenic bacteria, which may have therapeutic implications.