

Micronutrient modulation of NF- κ B in oral keratinocytes exposed to periodontal bacteria

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Abstract

Chronic periodontal diseases are characterised by a dysregulated and exaggerated inflammatory/immune response to plaque bacteria. We have demonstrated previously that oral keratinocytes up-regulate key molecular markers of inflammation, including NF- κ B and cytokine signalling, when exposed to the periodontal bacteria *Porphyromonas gingivalis* and *Fusobacterium nucleatum* *in vitro*. The purpose of the current study was to investigate whether α -lipoic acid was able to abrogate bacterially-induced pro-inflammatory changes in the H400 oral epithelial cell line. Initial studies indicated that α -lipoic acid supplementation (1–4 mM) significantly reduced cell attachment; lower concentrations (<0.5 mM) enabled >85% cell adhesion at 24 h. While a pro-inflammatory response, demonstrable by NF- κ B translocation, gene expression and protein production was evident in H400 cells following exposure to *P. gingivalis* and *F. nucleatum*, pre-incubation of cells with 0.5 mM α -lipoic acid modulated this response. α -Lipoic acid pre-treatment significantly decreased levels of bacterially-induced NF- κ B activation and IL-8 protein production, and differentially modulated transcript levels for IL-8, IL-1 β , TNF- α and GM-CSF, TLR2, 4, 9, S100A8, S100A9, lysyl oxidase, NF- κ B₁, HMOX, and SOD2. Overall, the data indicate that α -lipoic acid exerts an anti-inflammatory effect on oral epithelial cells exposed to periodontal bacteria and thus may provide a novel adjunctive treatment for periodontal diseases.

Keywords

α -Lipoic acid, epithelium, *Fusobacterium nucleatum*, periodontitis, *Porphyromonas gingivalis*

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Introduction

Periodontitis is a ubiquitous chronic inflammatory disease that destroys the supporting structures of teeth; the most common form is chronic periodontitis which, if untreated, results in the breakdown of soft tissues and bone, leading, ultimately, to tooth loss. The disease-initiating factor is plaque bacteria present in a biofilm at, and below, the gingival margin;¹ however, the subsequent tissue damage that characterises this disease is the result of an aberrant and exaggerated host response within susceptible individuals.² The plaque biofilm is a complex ecosystem containing a wide diversity of bacteria, two Gram-negative anaerobes *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, which are widely regarded as key to disease progression.³ *Fusobacterium nucleatum* interacts with other early colonising bacteria enabling a 'bridge' for the development of a more mature and pathogenic biofilm⁴ that is

colonised subsequently by strict anaerobes, including *P. gingivalis*, which expresses a range of virulence factors and is strongly associated with periodontal disease pathogenesis.⁵

An aberrant host immune response has been reported in periodontitis, with neutrophils from patients demonstrating hyperactivity/reactivity² and their excessive production of reactive oxygen species (ROS) is believed to be partly responsible for the local tissue damage, exacerbated by reduced local tissue antioxidant defences.⁶ The mechanisms whereby

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neutrophils are recruited to, or cleared from, the periodontal tissues may also have an important bearing on the aetiology of this disease. The sulcular and junctional epithelia, in common with similar epithelia at other sites in the body, were originally thought to simply provide a passive physical barrier, protecting the host from the external environment. More recently, however, periodontal epithelium has been shown to be a key orchestrator of the inflammatory response to a colonising biofilm and certain bacteria present within.⁷ Indeed, recent work has demonstrated that periodontitis-associated bacteria, such as *P. gingivalis* and *F. nucleatum*, can initiate keratinocyte pro-inflammatory responses resulting in elevations in cytokine transcript and protein production *in vitro*.⁸ Central to the pro-inflammatory response is NF- κ B, a key REDOX-sensitive transcription factor activated by increases in intracellular oxidative stress caused by a wide range of factors, including bacterial stimulation,^{8,9} resulting in downstream changes in gene expression and cytokine production.

α -Lipoic acid (ALA) (also known as α -lipoate or thiocytic acid) is a naturally-occurring di-thiol present in a wide range of food substances, including meats, such as liver, kidney and heart, as well as vegetables, such as spinach and broccoli. Recently, ALA has attracted considerable attention owing to its varied antioxidant actions. ALA is able to reduce levels of oxidative stress by (i) direct free radical scavenging and (ii) regeneration of other intracellular antioxidants,^{10,11} most notably glutathione—a key antioxidant in maintaining cellular REDOX status.¹²

Owing to its ability to boost levels of intracellular glutathione, ALA is able to regulate NF- κ B activation.¹³ ALA has undergone extensive clinical trials and has been shown to have efficacy in the treatment of several chronic inflammatory diseases, for example diabetes and cardiovascular disease.^{14,15} ALA therefore represents a potential candidate for modulation of NF- κ B and the associated pro-inflammatory activity of periodontal epithelium. Thus, the aim of this study was to investigate the effect of ALA on the pro-inflammatory response of oral keratinocytes exposed to periodontal bacteria by analysing NF- κ B activation, gene expression and cytokine production.

Materials and methods

Bacterial culture and suspensions

Escherichia coli LPS (serotype 026:B6; Sigma, Poole, UK) was reconstituted with cell culture growth media (DMEM; Invitrogen, Paisley, UK) to produce a stock solution (250 μ g/ml) which was stored at -20°C prior to use. *Porphyromonas gingivalis* (ATCC33277, isolated from the human gingival sulcus) and *F. nucleatum* (ATCC10953, isolated from inflamed human gingivae)

were grown in broth culture at 37°C anaerobically, as described previously.⁸ Cell suspensions were centrifuged, and the bacterial pellet washed three times using PBS, heat-inactivated (100°C for 10 min) and suspended in PBS to give a final concentration of 4×10^8 cells/ml. Heat inactivation was confirmed by plating and culturing experiments. Bacterial suspensions were stored at -20°C prior to use for keratinocyte exposure.

Cell culture

Details of cell culture methods were as described previously.^{8,16} Cells were grown in a range of plastic-ware, including flasks, 96-well plates and Petri dishes (including some containing multi-well glass slides). H400 cells (an immortal cell line derived from an oral squamous cell carcinoma of the alveolar process in a 55-year-old patient) were cultured in DMEM (Invitrogen) supplemented with 10% FCS (Labtech, Ringmer, UK), 4 mM glutamine (Sigma) and 0.5 μ g/ml hydrocortisone (Sigma). All cells were grown in an atmosphere of 5% CO_2 at 37°C . Once cells were seeded they were cultured for 4 d prior to media change and subsequently passaged on d 7. All experiments were performed between passages 4 and 20 using sub-confluent cell monolayers and standard DMEM media supplemented with growth factors (FCS, glutamine and hydrocortisone), which are used routinely for H400 cell culture to promote viability and proliferation.^{8,16} While hydrocortisone is known to have anti-inflammatory actions, under the conditions and concentrations we have employed this action is not particularly evident, and its inclusion is rather to enhance cell viability and proliferation in our culture system.^{8,16} In addition, media is supplemented frequently with antibiotics which we consider a potential confounding factor of H400 cell bacterial stimulation and not representative of the *in vivo* situation. Thus, all experiments reported in this article were performed in the absence of antibiotic supplementation.

ALA

A 0.06 M ALA (Sigma) stock solution was prepared in 0.029 M sodium hydroxide. The stock solution was stored at -20°C in aliquots prior to use.

Effect of ALA on cell adherence

Cells were pre-incubated with ALA (0.0625–4 mM) or vehicle control for 24 h to determine effects on cell adherence. Following incubation culture media was removed, cell monolayers were washed with 10 ml PBS and incubated with trypsin-EDTA for 10 min with regular agitation to ensure complete breakdown of the monolayer. The reaction was subsequently stopped by using fresh cell culture media.

Resulting cell suspensions were centrifuged and the cell pellet re-suspended in fresh culture media before cell counts were performed using a haemocytometer (modified Neubauer).

Effect of ALA on NF- κ B nuclear translocation in LPS stimulated keratinocytes

Escherichia coli LPS, a validated activator of NF- κ B nuclear translocation in various cell types, was used as a known positive control prior to testing relevant periodontal pathogens in our test system. H400 cells cultured on glass slides within Petri dishes were pre-incubated with ALA (0.25–2 mM) or vehicle control for 24 h prior to exposure to *E. coli* LPS (10 μ g/ml; 1 h) or vehicle control. Slides were then washed briefly in PBS, shaken to remove excess PBS and processed for immunocytochemical staining for NF- κ B and manual assessment of percentage translocation, as described in the following sections.

Effect of ALA on keratinocytes stimulated with periodontal bacteria

H400 cells were pre-incubated for 24 h in 0.5 mM ALA prior to exposure to *P. gingivalis* (ATCC33277) or *F. nucleatum* (ATCC10953). Periodontal bacteria were incubated with H400 cells at a concentration of 1×10^9 ml (equivalent to approximately 100 bacteria per epithelial cell) for 1 h. This ratio of bacteria (1:100, epithelial cell to bacteria) was comparable with previous studies which indicate the maximum number of bacteria associated with epithelial cells in the periodontal pocket, thereby providing clinical relevance.¹⁷ Cells or supernatants were analysed subsequently for NF- κ B nuclear translocation by high content analysis, gene expression by sq-RT-PCR and IL-8 protein by ELISA.

Immunocytochemistry of NF- κ B translocation

Cell monolayers were fixed using dry acetone for 10 min at room temperature (20–25°C), air dried and stained by immunohistochemistry. The staining protocol employed a MAb to the NF- κ B p65 subunit (clone F-6, diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and followed a biotin–streptavidin immunoperoxidase technique (StrAviGen, Biogenex, San Ramon, CA, USA) as reported previously.⁸ The resulting bound peroxidase was visualised using 3,3'-diaminobenzidine reagent for 5 min and counter-stained with Meyer's haematoxylin before mounting in Xam. Slide-washing and dilution of reagents were performed using 0.01 M PBS, pH 7.6. Positive- and negative-staining controls were included, which consisted of replacement of the NF- κ B monoclonal with Ki67 (clone MM1, diluted 1:100; NovacastraTM, Vision Biosystems, Newcastle, UK) as a positive

control and replacement of NF- κ B Ab with PBS or a MAb with irrelevant specificity, but the same immunoglobulin isotype as a negative control.

Quantification of NF- κ B translocation was performed on cell monolayers using a microscope (Leica, Wetzlar, Germany) fitted with an eyepiece graticule (10 \times 10 0.01 mm² squares) and viewed at 100 \times magnification. Analysis using Hunting curves indicated that counting of 30 randomly selected fields provided representative cell counts. For cells to be classified as demonstrating NF- κ B activation (positive), nuclear staining for p65 was required with no residual cytoplasmic staining evident. The total and mean numbers of positive cells were determined to calculate the percentage of cells demonstrating NF- κ B activation.

High content analysis of NF- κ B nuclear translocation

H400 cells were cultured in 96-well plates (Corning, Amsterdam, The Netherlands) to semi-confluence and either pre-incubated with 0.5 mM ALA for 24 h or vehicle (negative control) prior to exposure to *F. nucleatum*, *P. gingivalis* or PBS (negative control). Cultures were then washed with PBS and fixed using 1% formaldehyde solution for 20 min, washed again with PBS before filling wells with PBS. Plates were stored at 4°C prior to staining. Cells were incubated with the MAb NF- κ B p65 subunit (clone F-6 diluted 1:100; Santa Cruz) for 1 h at room temperature, washed and incubated with secondary Ab [anti-STAT1 rabbit polyclonal immunoglobulin (Santa Cruz)]-conjugated to a fluorescent label (Santa Cruz) for 1 h. Detergent buffer (Cellomics, Reading, UK; 200 μ l) was added to the wells for 15 min. Wells were then washed with PBS before further detergent buffer was added prior to analysis. Stained cultures were analysed using an ArrayScan imaging cytometer (Cellomics) with ArrayScan II data acquisition software (Cellomics) used for automated quantification of levels of cytoplasmic/nuclear NF- κ B staining.

Semi-quantitative RT-PCR analysis

RNA was extracted from H400 cell cultures using a commercially-available kit (RNeasy[®], Qiagen, Crawley, UK) and reverse transcription was performed using the Omniscript RT kit (Qiagen) to generate single-stranded cDNA as described previously.⁸ Semi-quantitative RT-PCR assays were performed using the RedTaq PCR system (Sigma). The reaction mixture consisted of 50 ng of single-stranded cDNA, 12.5 μ l RedTaq ready reaction mix, 10.5 μ l dH₂O, 1 μ l of 25 μ M forward and reverse primer. Table 1 provides primer sequences and reaction conditions. The resulting PCR mixture was amplified in a thermal cycler (Master cycler Gradient; Eppendorf, Stevenage, UK). The initial denaturing step was for 5 min at 94°C which was

Table 1. Details of primer sequence and semi-quantitative RT-PCR conditions. All DNA sequences are shown in the 5' to 3' orientation.

| Gene | Symbol | Accession Number | Sequence | T _m (°C) | Product (bp) | Cycle no. |
|--|--------------------|------------------|--|---------------------|--------------|-----------|
| Toll-like receptor 2 | TLR2 | NM_003264.3 | F-GAT GCC TAC TGG GTG GAG AA R-CGC AGC TCT CAG ATT TAC CC | 61 | 392 | 31 |
| Toll-like receptor 4 | TLR4 | NM_138444.2 | F-AAC CAT CCT GGT CAT TCT CG R-CGG AAA TTT TCT TCC CGT TT | 61 | 315 | 36 |
| Toll-like receptor 9 | TLR9 | AB045180.1 | F-CTG CGT CTC CGT GAC AAT TA R-GTC CTG TGC AAA GAT GCT GA | 61 | 443 | 36 |
| NF-κB ₁ | NF-κB ₁ | NM_003998 | F-CCT GGA TGA CTC TTG GGA AA R-CTT CGG TGT AGC CCA TTT GT | 61 | 366 | 26 |
| Tumour necrosis factor-α | TNF-α | NM_000594.2 | F-AAG AAT TCA AAC TGG GGC CT R-GGC TAC ATG GGA ACA GCC TA | 60 | 402 | 38 |
| Interleukin-1beta | IL-1B | NM_000576.2 | F-TCC AGG GAC AGG ATA TGG AG R-TTC TGC TTG AGA GGT GCT GA | 60 | 292 | 28 |
| Interleukin-8 | IL-8 | NM_000584.2 | F-TAG CAA AAT TGA GGC CAA GG R-GGA CTT GTG GAT CCT GGC TA | 60 | 204 | 30 |
| Granulocyte macrophage colony stimulating factor | GMCSF | M11220.1 | F-ACT ACA AGC AGC ACT GCC CT R-CTT CTG CCA TGC CTG TAT CA | 60 | 303 | 31 |
| Monocyte Chemoattractant Protein-1/CCL2 | MCP-1/CCL2 | NM_002982.3 | F-GCC TCC AGC ATG AAA GTC TC R-GCT GCA GAT TCT TGG GTT GT | 60 | 339 | 35 |
| Heme oxygenase 1 | HMOX1 | NM_0002133.1 | F-AAC CTC CAA AAG CCC TGA GT R-CAC CCC AAC CCT GCT ATA AA | 61 | 207 | 32 |
| S100 calcium binding protein A8 | MRP8/S100A8 | BC005928 | F-ATT TCC ATG CCG TCT ACA GG R-CAG CCT CTG GGC CCA GTA AC T C | 61 | 232 | 30 |
| S100 calcium binding protein A9 | MRP14/S100A9 | NM_002965.2 | F-CAG CTG GAA CGC AAC ATA GA R-TCA GCA TGA TGA ACT CCT CG | 61 | 228 | 23 |
| CCL20 | CCL20/MIP-3α | NM_004591.1 | F-GCA AGC AAC TTT GAC TGC TG R-CAA GTC CAG TGA GGC ACA AA | 60 | 341 | 27 |
| Lysyl oxidase | LOX | NM_002317 | F-ACA GGG TGC TGC TCA GAT TT R-CCA GGT AGC TGG GGT TTA CA | 61 | 381 | 24 |
| Superoxide dismutase 2 | SOD2 | S77127 | F-TGG AGG CAT CTA GTG GAA AAA R-CCC AGT CTC TCC CCA TTA CA | 61 | 305 | 34 |

T_m, annealing temperature (°C); bp, base pairs; (F), forward primer; (R), reverse primer.

then followed by amplification cycles comprising 94°C for 20 s, 60–61°C for 20 s and 72°C for 20 s, (see Table 1 for cycle number and annealing temperature per assay) with a final 10 min extension at 72°C. Six microlitres were removed from amplified reactions and separated on a 1.5% agarose gel (containing 0.5 µg/ml ethidium bromide; Sigma). The resulting gel was visualised under ultraviolet illumination using the EDAS 120 system (Kodak, Hemel Hempstead, UK) and the captured image was imported into AIDA image analysis software (Fuji, Bedford, UK). The volume density of amplified products was normalised against GAPDH housekeeping expression levels. All semi-quantitative RT-PCR experiments were performed in duplicate and representative graphs plotted.

ELISA

A commercially-available sandwich ELISA kit (IDS Ltd, Boldon, UK) enabling sensitive and specific detection/quantification was used to determine levels of IL-8 present in cell culture media. Culture media was filtered using a 0.2 micron filter (Millipore, Watford, UK) to remove cell debris prior to analysis. One hundred microlitres of standard or sample was added to wells and processed following the manufacturer's protocol. Absorbance was read at 450 nm (Spectrophotometer, Jenway, Stone, UK). Known IL-8 concentrations were used to generate a standard curve from which levels of IL-8 from the experimental samples (pg/ml) were determined. The detection limit for the assay was 25 pg/ml.

Results

Determination of ALA concentrations to be used in experiments using periodontal bacteria

As ALA culture supplementation has been reported previously to modulate NF-κB activation¹⁸ and initial experimentation indicated an ability to disrupt cell adhesion, dose-response experiments were performed to identify suitable concentrations for use with the H400 cell line. Data demonstrated that with increasing concentrations of ALA, adherent cell numbers decreased dose-dependently and ALA concentrations greater than 0.5 mM resulted in less than 50% cell adherence at 24 h (Figure 1). Reports in the literature indicate the ability of ALA to reduce expression of adhesion molecules,¹⁹ which may explain our adhesion data. Further investigation of H400 cells exposed to ALA indicated that non-adhered cells remained viable (Trypan blue exclusion staining) and after washing (to remove ALA), were able to re-adhere to culture plasticware and proliferate normally (data not shown). Combined data indicated that the use of ≤0.5 mM ALA was the most appropriate for subsequent experimentation.

To determine the effect of ALA on NF-κB translocation under simulated pro-inflammatory conditions in H400 cells, cultures were pre-incubated with a range of ALA concentrations (0–2 mM) for 24 h and then exposed to *E. coli* LPS (10 µg/ml) for 1 h. In these initial experiments *E. coli* LPS was used as this represents a well-characterised activator of the NF-κB pathway²⁰ and has been used previously to explore the modulatory effects of ALA in other cellular systems.²¹ Cell monolayers were stained immunocytochemically and manual counts were performed to determine the percentage of cells exhibiting nuclear translocation of NF-κB. Data demonstrated a dose-dependent inhibition of levels of NF-κB nuclear translocation with increasing levels of ALA, with 0.5 mM and 0.25 mM ALA supplementation resulting in a 78% and 59% reduction in NF-κB activation respectively (Figure 2). Based on these cell adherence and NF-κB activation studies further experiments investigating the effects of ALA on keratinocyte responses to periodontal bacteria were performed with 0.5 mM ALA.

NF-κB nuclear translocation and expression regulation by periodontal bacteria and ALA

To determine the kinetics of NF-κB nuclear translocation in H400 cells, using the periodontal bacterial stimuli *P. gingivalis* and *F. nucleatum*, high content analysis was performed. Initial experiments aimed to identify a suitable time-point for assay of NF-κB activity in H400 cells exposed to periodontal bacteria, which that demonstrated NF-κB translocation appeared to peak at different time-points for *P. gingivalis* (~60 min) and *F. nucleatum* (~100 min) (Figure 3). Subsequent experiments utilised 60 min of exposure for both *P. gingivalis* (~100% stimulation) and *F. nucleatum* (~84% stimulation) to enable comparison between bacteria and consistency with work published previously.⁸ These experiments demonstrated that NF-κB translocation was stimulated significantly by both *P. gingivalis* ($P=0.0016$) and *F. nucleatum* ($P=0.02$; Figure 4) compared with un-stimulated controls. Furthermore, cells pre-incubated with ALA (0.5 mM for 24 h) prior to bacterial exposure demonstrated significantly reduced levels of NF-κB activation when compared with stimulated cells that had not been pre-treated with ALA (*P. gingivalis*, $P=0.0026$; *F. nucleatum*, $P=0.008$) (Figure 4).

Semi-quantitative RT-PCR was performed subsequently to assess transcript level regulation (Figure 5) in genes identified previously as being associated with the inflammatory response, bacterial recognition (TLR), epithelial protection, antioxidant defence and tissue repair, as well as transcripts which we have identified previously as being regulated differentially by *P. gingivalis* and *F. nucleatum*.⁸ IL-8, GM-CSF, TNF-α and IL-1β demonstrated increased levels of gene

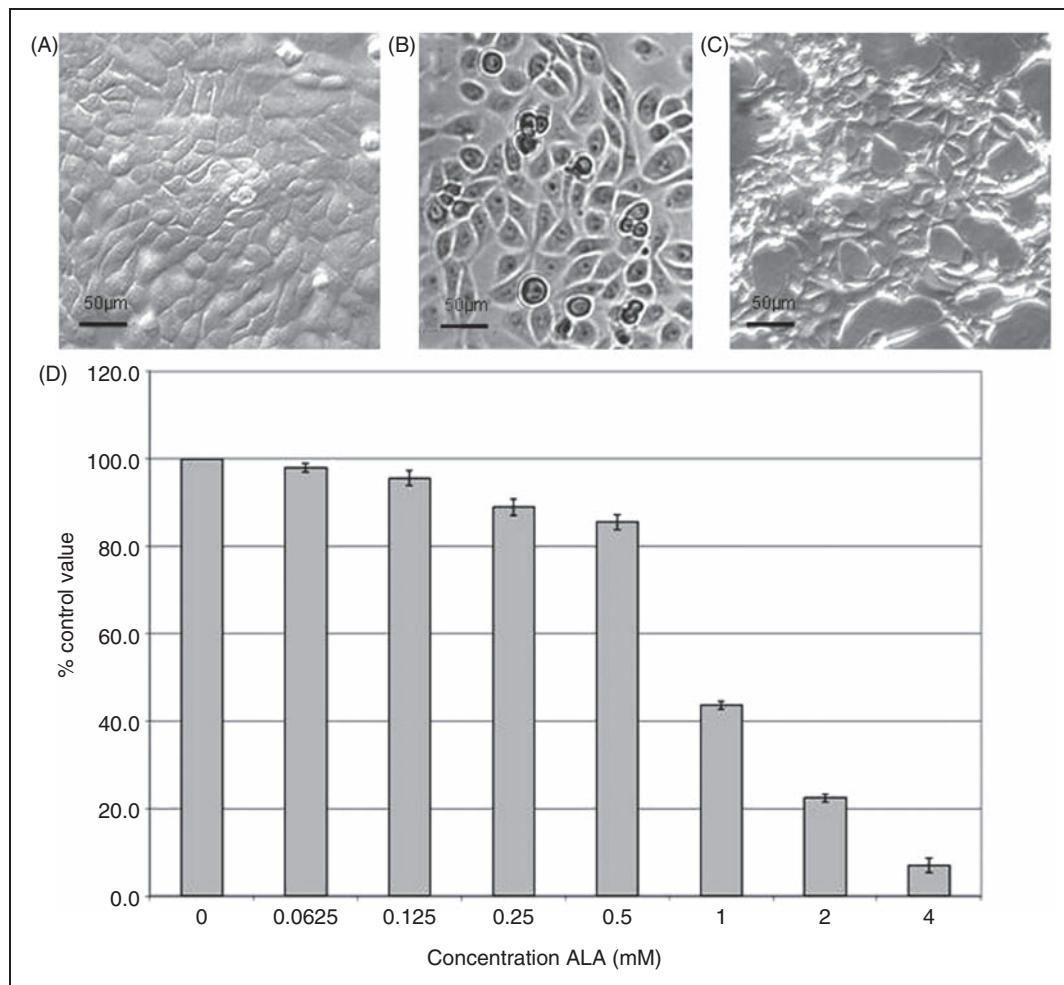


Figure 1. H400 cells incubated with (A) control solution (DMEM only; negative control) and (B) 0.5 mM α -lipoic acid for 24 h; (C) 4 mM α -lipoic acid for 24 h; (D) H400 cells incubated with a range of concentrations of α -lipoic acid for 24 h and counts of attached cells determined (mean value \pm standard deviation).

expression in H400 cells when exposed to both *P. gingivalis* and *F. nucleatum* when compared with un-stimulated controls. Pre-incubation with ALA (0.5 mM, 24 h) prior to bacterial exposure reduced these bacterially-induced transcript levels. In contrast, ALA pre-treatment of H400 cell cultures resulted in significant increases in TLR4 and TLR9 expression, while TLR2 transcript levels were decreased minimally. Exposure to *F. nucleatum* stimulated increases in both S100A8 and S100A9 transcript levels in H400 cells compared with un-stimulated controls, while *P. gingivalis* exposure resulted in minimal transcript level change compared with un-stimulated controls. Pre-incubation with ALA substantially reduced these transcripts in both stimulated and un-stimulated conditions for both these genes. Superoxide dismutase-2 (SOD-2) and haemoxygenase 1 (HMOX) are two transcripts important in antioxidant cellular defence. SOD-2 demonstrated increased expression following H400 exposure to *P. gingivalis* and *F. nucleatum*, while ALA pre-incubation affected these induced levels

minimally. HMOX gene expression was not increased following *F. nucleatum* stimulation of H400 cells; however, a clear increase in transcript level was evident following *P. gingivalis* stimulation, which was reduced to un-stimulated levels following H400 cell pre-incubation with ALA. Previous microarray analysis demonstrated down-regulation of lysyl oxidase (LOX) expression following H400 cell stimulation with *P. gingivalis* and *F. nucleatum*.⁸ In agreement with this finding data here demonstrated that H400 cell stimulation with *F. nucleatum* resulted in a minimal reduction in LOX expression while *P. gingivalis* stimulation resulted in a greater down-regulation of expression. However, ALA supplementation alone reduced LOX transcript levels significantly compared with both un-stimulated and *F. nucleatum*-stimulated cultures. A further minimal decrease was also detected in *P. gingivalis*-stimulated cultures. Pro-inflammatory related transcript, NF- κ B₁, which we have demonstrated previously to be increased in H400 cells in response to *P. gingivalis* or *F. nucleatum*,⁸ were further characterised in this

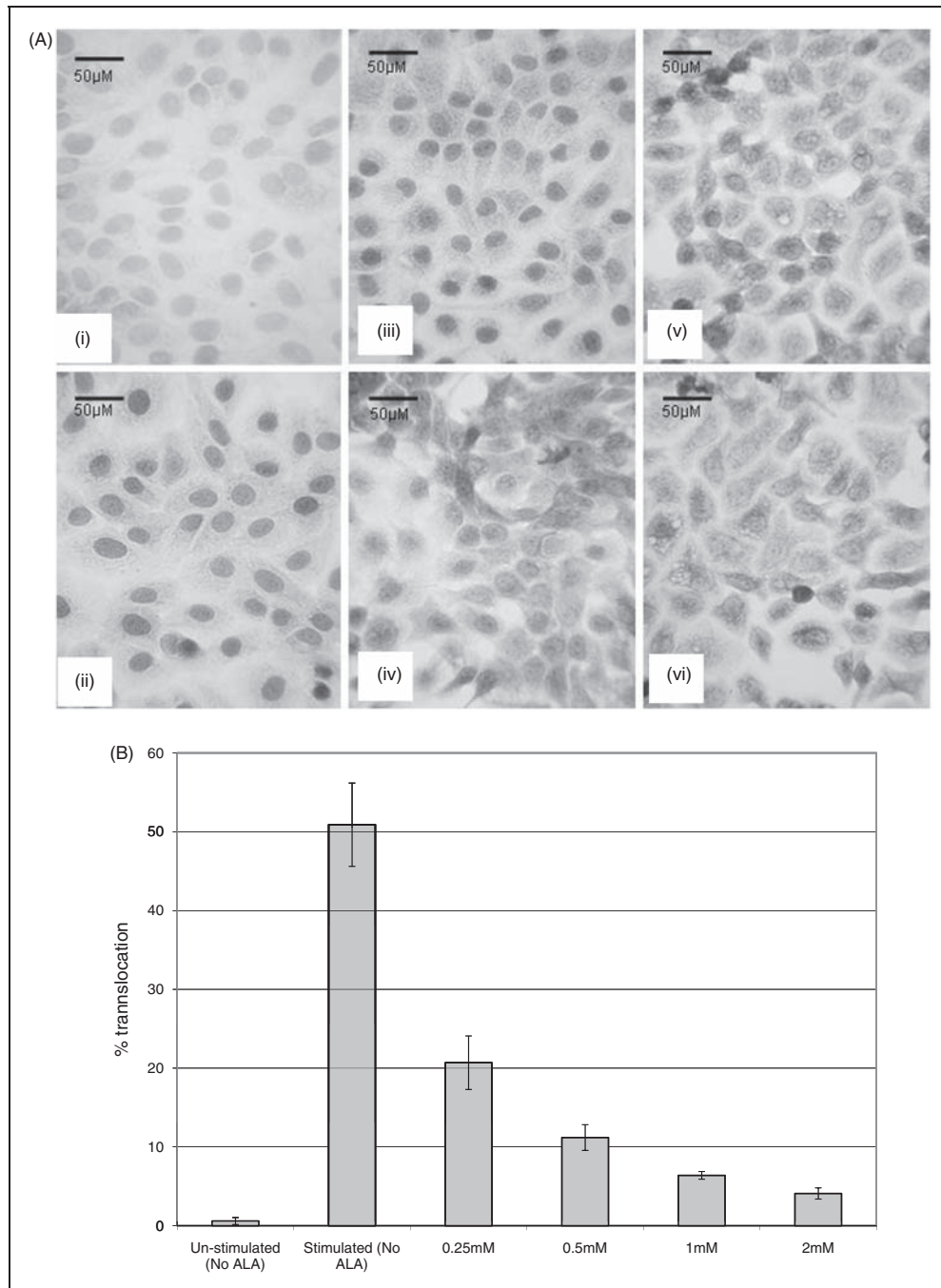


Figure 2. Analysis of H400 cells pre-incubated with a range of α -lipoic acid concentrations for 24 h prior to stimulation with *E. coli* LPS (1 h; 10 μ g/ml) and immunocytochemically assayed for NF- κ B translocation. (A) Images of stained cultures including (i) negative-staining control (primary Ab replaced with PBS), (ii) vehicle (DMEM) containing no ALA, (iii) 0.25 mM ALA, (iv) 0.5 mM ALA, (v) 1.0 mM ALA, and (vi) 2.0 mM ALA. Nuclear counterstaining has been used and scale bars are shown. Cytoplasmic localisation is progressively more pronounced as α -lipoic acid concentrations increase. (B) Graphical analysis showing percentage of total cell numbers exhibiting nuclear NF- κ B localisation (mean \pm SD).

model system. Concomitantly, increased expression was detected for NF- κ B₁ following cellular stimulation with both *P. gingivalis* and *F. nucleatum*. Bacterially-induced levels of this transcript were decreased following ALA pre-treatment of cultures.

While transcriptional regulation in H400 cells by ALA was demonstrated further confirmation that these changes

translated to effects on functional protein levels was investigated. Subsequently, analysis of the levels of pro-inflammatory cytokine, interleukin (IL-8), a surrogate marker of NF- κ B activation demonstrated that stimulation with *P. gingivalis* and *F. nucleatum* increased secreted protein levels of IL-8, while pre-incubation of cells with ALA significantly reduced these stimulated levels (Figure 6).

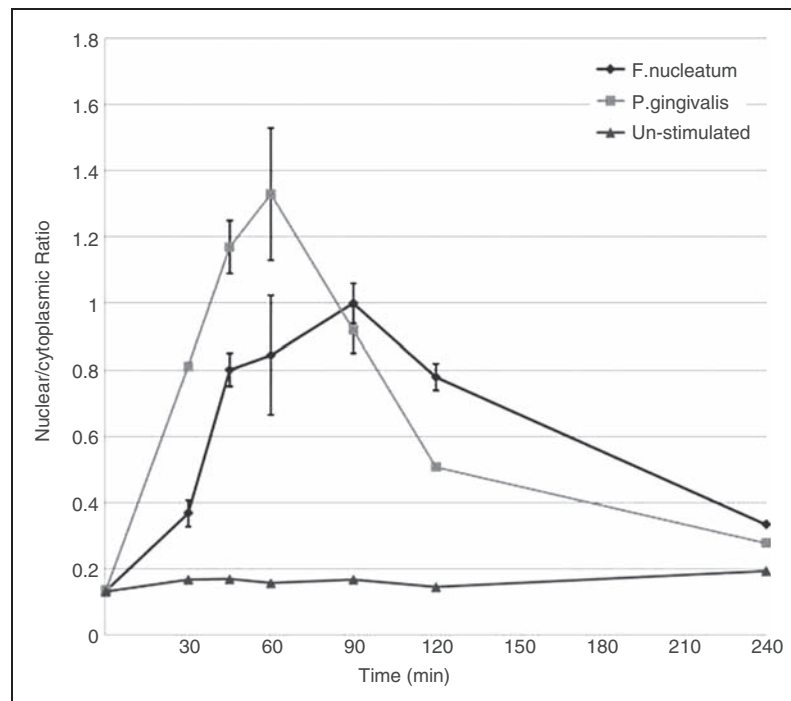


Figure 3. NF- κ B translocation kinetics for H400 cells exposed to *P. gingivalis* and *F. nucleatum* determined by high content analysis over a 240-min time period. Levels of translocation are shown as nuclear:cytoplasmic ratios (mean \pm SD, $n = 3$).

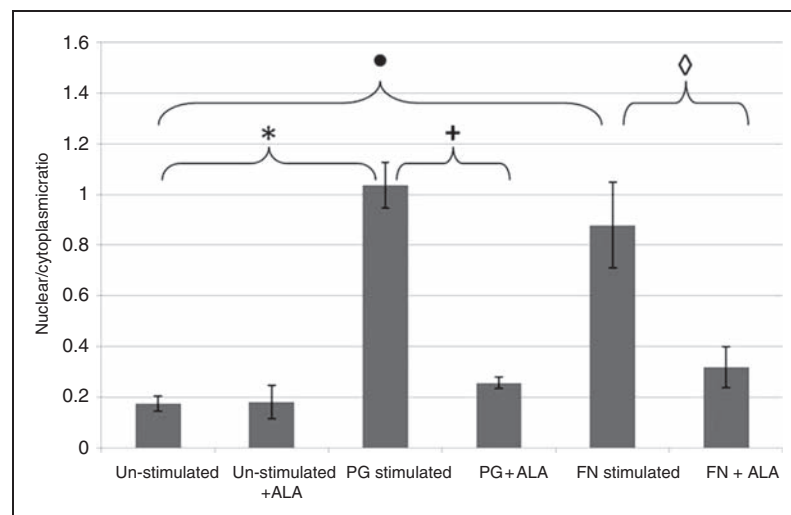


Figure 4. High content immunocytochemical analysis of NF- κ B in H400 cells exposed to *P. gingivalis* or *F. nucleatum* \pm α -lipoic acid ($n = 3$, mean \pm SD). Cultures were pre-incubated with 0.5 mM ALA for 24 h prior to exposure to bacteria for 1 h. Symbols indicate statistical significant differences between exposure groups (* $P = 0.0016$; + $P = 0.0026$; • $P = 0.02$; ◇ $P = 0.008$).

Discussion

Current evidence regarding periodontal disease pathogenesis indicates that the substantive tissue damage seen in patients is a result of a dysregulated, non-resolving inflammation in response to the plaque biofilm²² associated with oxidative stress²³ and elevated neutrophil reactive oxygen species release² characteristic of the periodontitis phenotype. Emerging data indicate

that, initially, the oral epithelium detects the presence of periodontal bacteria⁷ and, plays an important role in orchestrating the subsequent pro-inflammatory response. The regulation of epithelial responses therefore provides a point for potential therapeutic intervention and management of this disease. The NF- κ B signalling pathway is a key molecular regulator of the cellular inflammatory response and is REDOX sensitive; therefore, subsequent changes in intracellular

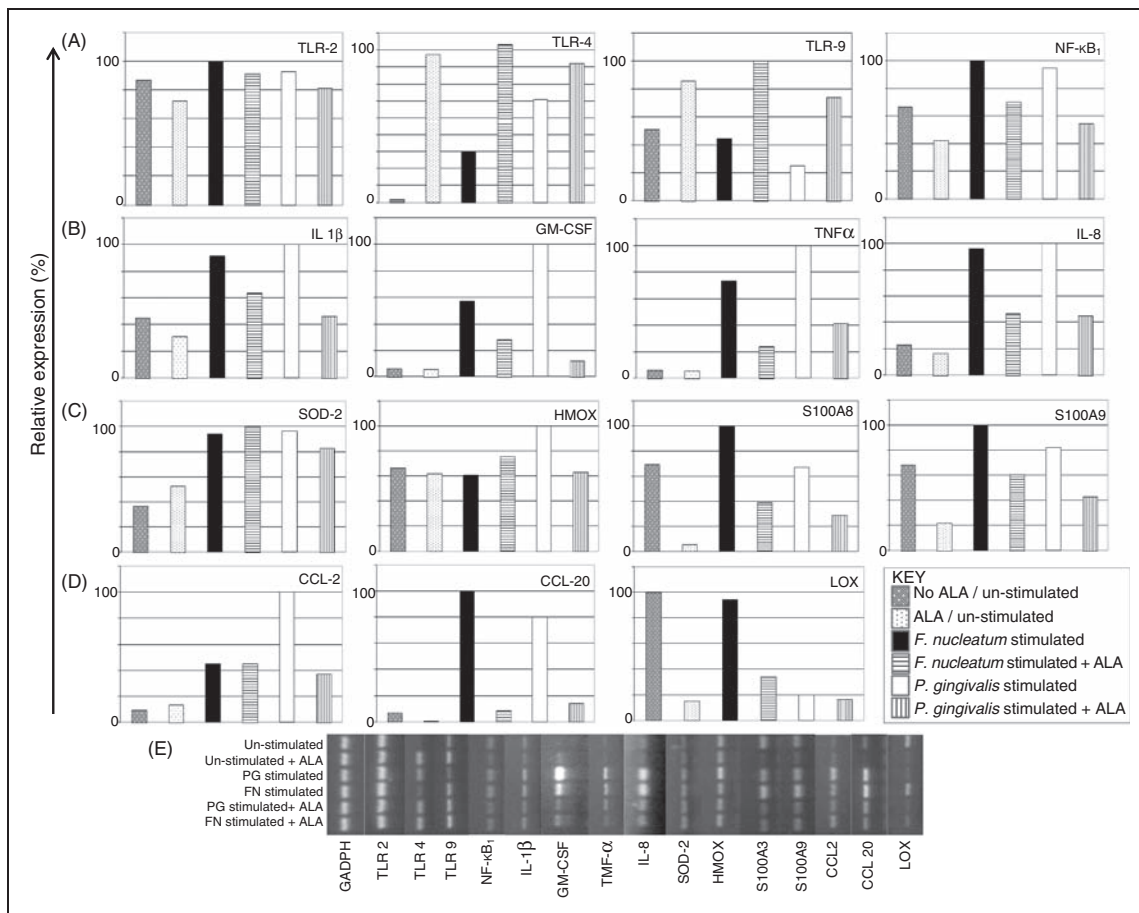


Figure 5. Gene transcription changes as determined by sq-RT-PCR analysis in H400 cells exposed to *P. gingivalis* (1 h), *F. nucleatum* (1 h) or media (negative control) and pre-incubated with 0.5 mM α -lipoic acid for 24 h. (A) Genes associated with bacterial recognition and the NF- κ B transcription pathway; (B) pro-inflammatory cytokines; (C) genes associated with epithelial protection; (D) cell recruitment and tissue repair associated genes; (E) representative gel images. All data shown is representative of at least duplicate analyses where comparable results were obtained.

oxidative stress levels regulate pathway activation and result in downstream sequelae, including cytokine production.²⁴ This pathway therefore provides an ideal target for modulation of the inflammatory response and, as the natural di-thiol ALA is reported to boost intracellular antioxidant capacity, it has been identified as an ideal candidate for modulating epithelial-driven inflammation. This study investigated the effects of ALA on key stages in periodontal pathogen stimulated epithelial cell activation, including bacterial component recognition via TLRs, NF- κ B signalling, transcriptional responses and cytokine production. The expression of three key TLRs were investigated and data demonstrated that ALA pre-treatment may result in increases in keratinocyte expression of these molecules which, hypothetically, may have translated to increases in the cells' sensitivity and ability to detect bacteria. Conversely, however, epithelial cell pre-incubation with ALA with subsequent bacterial stimulation resulted in reduced levels of NF- κ B activation, suggesting that increases in TLR transcript levels are not directly translated to increases in inflammation owing

to ALA's ability to modulate activation of NF- κ B. These findings are in agreement with data from previous studies which have analysed a range of other cell types and have shown that ALA pre-treatment reduces the initiation and propagation of the inflammatory response.^{25–28}

ALA is important in regenerating intracellular antioxidants, including glutathione, which is central to maintaining cellular REDOX status.^{10–12} Mechanistically, it has been shown that ALA is internalised into the cell and converted to its more active antioxidant form, dihydrolipoic acid (DHLA).²⁹ Preliminary experiments have demonstrated that incubation of ALA with H400 cells enhances its antioxidant capacity, as determined using an enhanced chemiluminescence assay³⁰ (data not shown), indicating that H400 cells are able to metabolise ALA to its more active antioxidant form and suggesting that our observations are likely explained by ALA boosting intracellular antioxidant levels in H400 cells.

Interestingly, LOX gene expression appeared to be reduced following exposure to *F. nucleatum* and

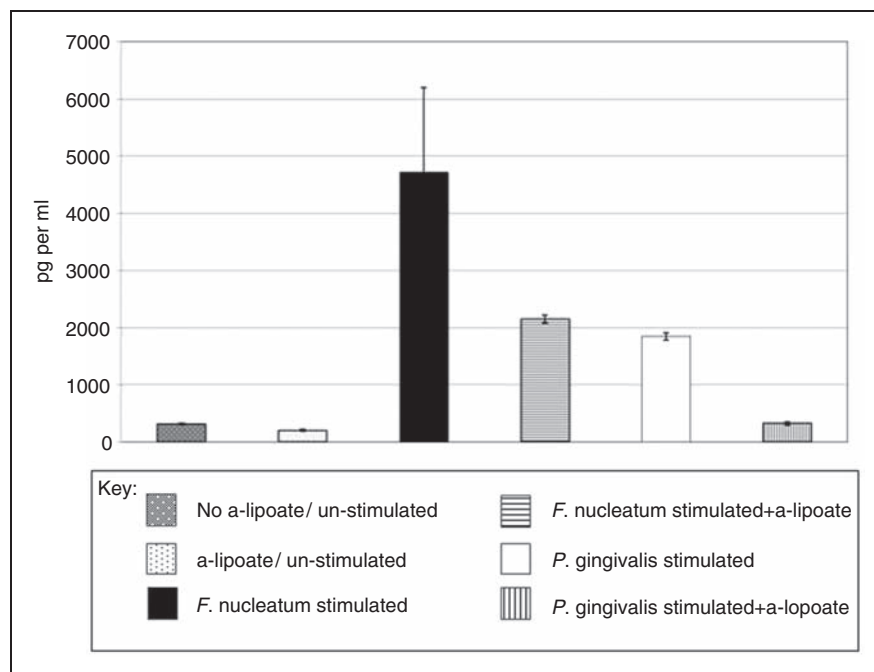


Figure 6. Secreted IL-8 levels (pg/ml) as determined by ELISA in H400 cell cultures pre-incubated with 0.5 mM α -lipoic acid for 24 h and exposed to *P. gingivalis* (1 h), *F. nucleatum* (1 h) or media alone. Combinations of culture exposure conditions are shown in the key. Mean \pm SD.

P. gingivalis. LOX is a copper-dependent enzyme important in epithelial function, development and repair, and functions by cross-linking elastin and collagen.³¹ In periodontitis breakdown of these tissues is associated with disease progression. Therefore, reduction in expression of LOX post *P. gingivalis* and *F. nucleatum* stimulation may play a role in the pathogenic mechanism induced by these bacteria and conceivably lead to the periodontal tissue breakdown and frustrated tissue healing characteristic of this disease. LOX gene expression was also reduced in the presence of ALA; this may be due to the copper-chelating properties of ALA, an essential co-factor in the action of this enzyme.³² ALA has been used in a range of chronic inflammatory diseases and reports in other systems, including skin²⁵ and gastric ulcers,³³ have suggested that ALA enhances healing. Further studies are therefore needed to investigate functional protein production in response to ALA supplementation.

Genes that have a role in cellular protection, neutrophil recruitment and activation (MRP-8/S100A8 and MRP-14/S100A9) were potentially reduced in bacterially-stimulated cells owing to pre-incubation with ALA. While these data indicate that ALA supplementation may reduce cell protection mechanisms following bacterial challenge, these reductions may also result in reduced neutrophil chemotaxis and activation subsequently modulating the pro-inflammatory response. In addition, recent work³⁴ has demonstrated that the increased expression of these molecules associates with ROS-mediated transcription pathway activation, therefore their down-regulation in response to ALA

further supports its anti-inflammatory action. Pre-incubation of the H400 cells with ALA resulted in a potential decrease in transcript levels of NF- κ B which may equate to reduced cellular levels of the translated protein which potentially diminishes the activation potential of this pathway. In further support of this, pre-incubation of H400 cells with ALA prior to stimulation with *P. gingivalis* and *F. nucleatum* potentially reduced protein transcription levels of the key neutrophil recruiting and activating cytokine, IL-8. As this molecule is implicated in the induction and propagation of a variety of chronic inflammatory responses the ability of ALA to modulate IL-8 production may have important ramifications for periodontal disease management. Pre-incubation of oral epithelial cells with ALA has now, for the first time, been shown to modulate periodontal bacterial induced NF- κ B activation, pro-inflammatory gene expression and cytokine production. It is, however, interesting to note that ALA does not abrogate the pro-inflammatory response, but rather attenuates it. Indeed, complete blocking of this key first-line host response to bacterial challenge may be detrimental as it may render the host susceptible to overwhelming bacterial invasion. Therefore, this characteristic of ALA has potentially important clinical implications; the aim of any new anti-inflammatory therapeutic agent would be to appropriately reduce exaggerated levels of inflammation in periodontitis patients and facilitate resolution of inflammation with minimal collateral tissue damage.

Our immunocytochemical and high-throughput data suggest increased levels of NF- κ B translocatory

activation with *P. gingivalis* compared with *F. nucleatum* stimulation. Thus, the finding of significantly higher epithelial secretion of IL-8 after *F. nucleatum* stimulation compared with *P. gingivalis* stimulation appears contrary to the NF- κ B data. The possible reasons for this disparity in data are unclear; however, it is possible that: (i) NF- κ B activation does not directly correlate with IL-8 levels, for example levels of activated transcripts may be significantly induced, but this may not translate to increased protein levels; and (ii) the kinetics of activation of NF- κ B likely influence IL-8 levels, which were measured 24 h after bacterial challenge. There is little published data on the kinetics of induction of NF- κ B over a 24 h time period. It is clear from our data that a different temporal activity profile exists for NF- κ B activity when stimulated by the two bacteria, with nuclear translocation being highest for *P. gingivalis* at 60 min but lower than *F. nucleatum* at 90 min and 120 min after. Such differences in temporal profile may explain why higher IL-8 levels are detected following *F. nucleatum* stimulation. In addition, while our data and that of others indicate that nuclear translocation of NF- κ B peaks at about 1 h after stimulation and is recycled by 4 h, it is not clear as to when nuclear translocation can be re-stimulated. It may, therefore, be feasible that different NF- κ B activation profiles exist for *F. nucleatum* and *P. gingivalis* stimulation within the 24-h time period, which may also explain the lack of correlation between 60-min NF- κ B nuclear translocation and IL-8 levels determined at 24 h.

ALA has been used widely for managing a range of chronic inflammatory diseases, including those which associate with periodontitis, such as diabetes;³⁵ however, its potential in managing periodontal disease has not been proposed previously. This study investigated the effects of this natural di-thiol on periodontal pathogen-stimulated oral epithelium in terms of NF- κ B activation, gene expression changes and cytokine production, utilising a previously well-characterised epithelial cell culture system.^{8,16} Data indicated the ability of periodontal bacteria (*F. nucleatum* and *P. gingivalis*) to stimulate a pro-inflammatory phenotype which was subsequently modified by pre-incubation of H400 cells with ALA. These data suggest that ALA supplementation, either via local or systemic delivery, may provide utility in managing periodontitis patients.

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