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Stimulants of Toll-like receptors 2 and 4 are elevated in saliva of periodontitis patients compared with healthy subjects

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Abstract

Aim: Because the absorption of stimulants of Toll-like receptor (TLR)2 and TLR4 from the gastrointestinal tract into the circulation has been proposed to promote the development of atherosclerosis and insulin resistance, we aimed to quantify the abundance of stimulants of TLR2 and TLR4 in human saliva.

Methods: A recently developed bioassay based upon measurement of NF-κB activation in TLR-deficient human embryonic kidney (HEK)-293 cells transfected with human TLR2 or TLR4 and calibrated with synthetic bacterial lipopeptide (Pam_3CSK_4) or *Escherichia coli* lipopolysaccharide (LPS), was used to establish the normal range of TLR stimulants in saliva of 20 healthy subjects and 20 subjects with periodontal disease.

Results: Median soluble stimulants of TLR2 and TLR4 were significantly higher in saliva of periodontitis patients compared with saliva of healthy subjects; 3450 *versus* 77 ng/ml Pam_3CSK_4 equivalents ($p < 0.0001$) and 138 *versus* 7 ng/ml LPS equivalents, respectively ($p < 0.0001$). Salivary TLR stimulant levels remained relatively stable in healthy subjects over several days. Six strains of oral Gram-negative bacteria, including *Tannerella forsythensis*, *Lysobacter enzymogenes*, *Prevotella intermedia*, *Prevotella oris* and *Porphyromonas gingivalis*, from a panel of nine examined did not stimulate TLR4-dependent signalling.

Conclusions: Elevated salivary TLR stimulants may represent a novel mechanism by which periodontitis increases the risk of developing cardiovascular disease and insulin resistance.

Key words: atherosclerosis; endotoxin; insulin resistance; lipopeptide; periodontitis; saliva; Toll-like receptors

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Although it has often been reported that periodontitis is associated with an increased risk of atherosclerosis and insulin resistance, the mechanisms underlying these associations are currently unclear (Beck et al. 2001, Tonetti 2009, Teeuw et al. 2010). One popular hypothesis is that periodontal disease may result in an increased frequency of transient endotoxaemias and bacteraemias, thereby raising systemic inflammatory tone and contributing to the development of these diseases (Geerts et al. 2002, Pussinen et al. 2004, Lockhart et al. 2008). However, an alternative mechanism we have recently proposed is that bacterial products present in the small intestine may be absorbed with dietary fat to promote low-grade systemic inflammation, thereby potentiating these diseases (Erridge et al. 2007a, Erridge 2008, 2009). This notion is supported by the observation that dietary fat promotes the absorption of intestinal lipopolysaccharide (LPS) into the circulation in humans and in animal models, where it may contribute to systemic low-grade inflammation and metabolic dysregulation (Cani et al. 2007, Erridge et al. 2007a, Laugerette et al. 2011). Notably, a small proportion of the endotoxin present

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in the small intestine has been shown to be packaged into chylomicrons with dietary fat, suggesting that the small intestine may be a major route for the translocation of fat-soluble bacterial products into the circulation (Ghoshal et al. 2009, Laugerette et al. 2011).

Such products may promote inflammatory signalling via their interaction with specific receptors of the innate immune system, of which the Toll-like receptors (TLRs) represent a major family. The 10 human TLRs serve to detect conserved ''pathogen-associated molecular patterns'' (PAMPs), which are present in microbial cells or viruses, but not host cells (Kumar et al. 2009). For example, TLRs 2, 4 and 5 detect bacterial lipopeptides, LPSs and flagellins, respectively, whereas TLRs 3, 7, 8 and 9 detect nucleic acid motifs (Kumar et al. 2009). Recent data suggest that in addition to their beneficial role in host defence, chronic low-grade stimulation of TLR signalling, particularly via TLR2 and TLR4, may also contribute to the development of a number of diseases that are promoted by chronic inflammatory processes. For example, experimental administration of TLR2 or TLR4 stimulants to mice leads to a significant acceleration of atherosclerosis (Mullick et al. 2005, Westerterp et al. 2007) and insulin resistance (Cani et al. 2007), whereas genetic deficiency in TLR2 or TLR4 protects against the development of these diseases (Michelson et al. 2004, Mullick et al. 2005, Poggi et al. 2007, Himes & Smith 2010).

As the commensal microbiota of the small intestine is relatively limited ($\sim 10^0$–10^6 CFU/ml), we reasoned that a significant proportion of the bacterial products present in the small intestine may derive from swallowed products of the oral microbiota rather than from the endogenous intestinal bacteria. To address this hypothesis, we applied a recently developed bioassay to quantify the relative biological activities of TLR stimulants in human saliva using human embryonic kidney (HEK)-293 cells transfected with specific TLRs and NF-κB reporter (Erridge 2010, Erridge et al. 2010). We then examined the profile of TLR2, TLR4 and TLR5 stimulants expressed by a panel of 13 major oral bacteria, and quantified the extent of shedding of such stimulants by a model enterobacterial organism to yield a first estimate of the relative contributions that may be made by oral and enteric bacteria to the PAMP content of the small intestine.

Materials and Methods

Ethics and informed consent

Subjects gave written informed consent for the study, which was approved by the University of Leicester College of Medicine Research Ethics Committee and by the Glasgow Dental Hospital and School Ethics Committee. All subjects were also informed that they had the right to withdraw from the study at any time.

Recruitment of subjects and sample collection

Minimally stimulated saliva ($\sim$ 4 ml) was collected from 20 healthy human volunteers by expectoration into sterile universal tubes, not $<$30 min. after eating, drinking or smoking. The controls comprised smokers and non-smokers who reported no history of periodontal disease, no bleeding on brushing and had between 27 and 32 teeth. Those having had extractions had teeth removed for orthodontic reasons only. Saliva samples from 20 age- and sex-matched patients with chronic adult periodontitis were also examined. Prospective patients were identified by screening the patient databanks of the Periodontal Department of Glasgow Dental Hospital and School for subjects who had presented for treatment with a Community Periodontal Index of Treatment Need (CPITN) score of 4 in at least one sextant and had then completed a course of periodontal treatment and were receiving supportive therapy as described earlier (Lappin et al. 2007). Saliva samples were promptly stored at -20°C and thawed immediately before analysis. Plaque indices were not measured because the patients adhered well to their instructions to brush their teeth, and as they had typically done so around 3 h before their visit to provide samples, this was not sufficient time for plaque to develop.

Inclusion criteria

The subjects with chronic periodontitis had at least 16 teeth, including at least four molars, and had, in different quadrants, at least two periodontal pockets $>$4 mm in depth, with a minimum of 2 mm attachment loss and reported brushing teeth at least twice daily.

Exclusion criteria

Patients were excluded if they presented with any other periodontal condition or systemic disease (e.g. diabetes or cardiovascular disease, etc.), if they were pregnant; or if they had received antibiotic therapy within the past 3 months; or if they had taken non-steroidal anti-inflammatory drugs in the past 6 weeks. Smokers who smoked fewer than 10 cigarettes per day and former smokers were also excluded.

Cell culture and reagents

HEK-293 cells were cultured in DMEM supplemented with 10% foetal calf serum (FCS, Sigma, Poole, UK). PAMP standards for assay calibration were, for TLR2, synthetic bacterial lipopeptide Pam_3CSK_4 (Invivogen, Toulouse, France), for TLR4, *Escherichia coli* LPS repurified by phenol–water re-extraction to remove TLR2-stimulating lipopeptide contaminants as described previously (Hirschfeld et al. 2000) and for TLR5, *Salmonella typhimurium* flagellin (Invivogen). Oxidized palmitoyl arachidonyl phosphatidyl choline (OxPAPC) was prepared by auto-oxidation in air for 72 h as described previously (Erridge et al. 2008).

Quantification of TLR stimulants in saliva samples

TLR stimulants were quantified in both whole heat-treated saliva (100°C for 10 min.), intended to reflect total bacteria-associated TLR stimulants, and in saliva diluted 1:10 in phosphate-buffered saline (PBS) and filter-sterilized (0.22 μm, Acrodisc, Port Washington, NY, USA), intended to reflect soluble TLR stimulants in human saliva. A recently developed bioassay based upon the measurement of NF-κB-dependent reporter activation in TLR-deficient HEK-293 cells transfected with human TLR2, TLR4/MD2 or TLR5, and calibrated with TLR2-, TLR4- or TLR5-stimulating standards (Erridge et al. 2010), was used to quantify TLR stimulants in saliva samples. Briefly, cells were plated in 96-well plates at 2×10^4 cells/well and transfected after 24 h using Genejuice (Novagen, Gibbstown, NJ, USA). Amounts of construct per well were 30 ng of human TLR2, TLR4 (co-expressing MD-2) or TLR5 (Invivogen), 30 ng of CD14 and 10 ng of firefly luciferase-reporter construct driven by the NF-κB-dependent E-selectin promoter (pELAM). Three days after transfection, cells were challenged in triplicate with heat-treated or sterile-filtered saliva samples diluted 1:100 in DMEM/1%FCS. In the same plate, an eight-point standard

curve was prepared using dilutions of Pam_3CSK_4 (100–0.032 ng/ml), *E. coli* LPS (100–0.032 ng/ml) or *S. typhimurium* flagellin (from 1000 to 15 ng/ml), in duplicate. After 18 h, NF-κB-dependent reporter expression was measured using Promega Dual-Glo reagent. Fold induction of reporter was calculated relative to cells cultured in medium alone and a standard curve was prepared by plotting fold NF-κB induction *versus* concentration for each standard PAMP. The relative biological activities of specific TLR stimulants in saliva were then calculated as ng/ml saliva, and are presented as a relative biological activity with respect to Pam_3CSK_4, LPS or flagellin, as described previously (Erridge et al. 2010). For example, results presented as 200 ng/ml lipopeptide equivalents indicate that 1 ml of saliva contains TLR2 stimulants with a capacity to stimulate TLR2 signalling equal to that of 200 ng of Pam_3CSK_4. The coefficient of variance of the assay averaged ~ 20%. PAMP standards did not induce signalling in cells expressing heterologous TLRs, or in cells transfected with CD14 alone (Erridge et al. 2008 and data not shown).

Bacterial strains used

Strains of bacteria examined were: *Aggregatibacter actinomycetemcomitans* (NCTC9709); *Campylobacter rectus* (DRWH); *Fusobacterium nucleatum* (NCTC10502); *Streptococcus sanguinis* (NCTC7163); *Streptococcus salivarius* (NCTC8018); *Tannerella forsythensis* (ATCC95137); *Lysobacter enzymogenes* (DSM1895); *Porphyromonas gingivalis* (NCTC11834); *Porphyromonas gingivalis* (W50); *Prevotella intermedia* (ATCC25611); *Prevotella oris* (ATCC 33573); *Peptostreptococcus micros* (NCTC11808); *Streptococcus mutans* (NCTC10449); *Pseudomonas aeruginosa* (PAC611) and *E. coli* K12 (ATCC 27325). Each strain was resuspended in saline to an absorbance at 600 nm of 1.0, equivalent to ~ 10^9 bacteria/ml, and heat-killed at 100°C for 10 min. before storage at −20°C before assay. The capacity of each organism to stimulate TLR2-, TLR4- or TLR5-dependent signalling at 10^7 bacteria/ml was then measured in HEK-293 cells transfected as described above. Positive controls for NF-κB activation were 10 ng/ml *E. coli* LPS, 10 ng/ml Pam_3CSK_4 (Pam3), 10 ng/ml *S. typhimurium* flagellin (Flag) or 10 μg/ml polyinosinic acid (PolyI:C) which stimulates NF-κB activation independently of TLR2, TLR4 or TLR5 in HEK-293 cells (Erridge et al. 2008). Results are reported as mean fold induction of NF-κB reporter in triplicate cultures relative to cells cultured in medium alone.

Growth of *E. coli* for measurement of TLR stimulants in conditioned media

In order to investigate the extent of shedding of soluble TLR2 and TLR4 stimulants by a model enterobacterial organism, cultures of *E. coli* K12 were grown in Luria broth (LB) from a starting density of ~ 1×10^8 bacteria/ml with shaking at 37°C. 1 ml aliquots were taken each hour for 4 h and optical density (OD) at 600 nm was measured to provide an estimate of bacterial growth (OD of 1.0 was assumed to reflect ~10^9 bacteria/ml). Aliquots taken at each timepoint were centrifuged (13,000 *g* for 5 min.), and the supernatant was filter-sterilized (0.22 μm, Acrodisc). Soluble TLR2 and TLR4 stimulants were then measured in each supernatant by bioassay as described above.

Statistics

Log_{10} transformation of TLR stimulant concentrations was performed to normalize the data distribution before analysis. Transformed TLR stimulant concentrations in saliva of healthy and periodontitis subjects were then compared using Student's *t*-test. ANOVA with Dunnett's or Tukey's post hoc test was used to compare TLR-dependent NF-κB activation induced by defined bacterial isolates, or saliva samples treated with TLR inhibitors, respectively. For comparison of TLR-stimulant levels between high and low responders, values were log-transformed and means of the pooled results from the 3 days examined were compared by Student's *t*-test. Pearson's r^2 was used to measure the correlation between variables. Differences were considered statistically significant at $p < 0.05$.

Results

Clinical data

The periodontitis patients (age 41 ± 2.5 years, 13F:7M, eight smokers) brushed at least twice daily, had a mean pocket depth of 2.84 (± 0.37) mm, number of teeth was 24.1 (± 1.6), number of sites with pocket depth >4 mm was 12.0 (± 10.4) and clinical attachment level was 3.53 (± 0.75) mm. As a measure of gingival inflammation, bleeding on probing was recorded within the patient group. This analysis revealed that on average 30% (± 10%) of sites bled on probing. All the healthy controls (age 38 ± 10.7 years, 14F: 6M, two smokers) had between 27 and 32 teeth (where orthodontic reasons accounted for missing teeth), had no history of periodontitis, reported no bleeding of gums on toothbrushing and brushed on average 1.9 times/day. All clinical data are presented as means ± SD.

Quantification of TLR stimulants in heat-sterilized human saliva

We chose to examine the concentrations of TLR stimulants in saliva samples in two ways. First, saliva samples were heat-sterilized to represent the total PAMP content present in saliva, including those attached to bacteria. Next, saliva diluted 1:10 in PBS was filter-sterilized to yield an extract intended to reflect the soluble PAMPs present in saliva. These two preparations were investigated separately as it was reasoned that soluble PAMPs may be more likely to be absorbed than PAMPs that remain attached to bacteria (Ghoshal et al. 2009).

In heat-treated saliva of healthy subjects, median TLR2-stimulant concentrations were 304 ng/ml (range 90–3540 ng/ml) lipopeptide equivalents, as measured relative to the biological activity of Pam_3CSK_4 synthetic lipopeptide standard (Fig. 1). Median TLR2 stimulants were 3640 ng/ml (range 52–14,300 ng/ml) lipopeptide equivalents in saliva from periodontitis patients ($p < 0.01$ *versus* healthy subjects). Median TLR4 stimulants measured in heat-treated saliva from healthy subjects were 55 ng/ml (range 25–182 ng/ml), as measured relative to the biological activity of *E. coli* LPS standard, and 325 ng/ml (range 67–6090 ng/ml) in saliva from periodontitis patients ($p < 0.001$ *versus* control saliva). Median TLR5 stimulants were 5.4 μg/ml (range 0.3–23 μg/ml) in healthy subjects and 32 μg/ml (range 1.1–98 μg/ml), relative to *S. typhimurium* flagellin, in periodontitis patients ($p < 0.001$ *versus* control saliva).

Quantification of soluble TLR stimulants in filter-sterilized human saliva

We next quantified the abundance of soluble stimulants of TLR2, TLR4 and TLR5 in filter-sterilized saliva. Median soluble TLR2 stimulants were 77 ng/ml (range 24–465 ng/ml) in healthy sub-

jects and 3450 ng/ml (range 44–35,100 ng/ml) in periodontitis patients ($p < 0.001$). Median soluble TLR4 stimulants were 7 ng/ml (range 4–99 ng/ml) in healthy subjects and 138 ng/ml (range 77–2020 ng/ml) in periodontitis patients ($p < 0.001$). Soluble TLR5 stimulants were not detectable in the sterile-filtered saliva samples, suggesting that flagellin may remain predominantly attached to bacteria or in the multimeric form rather than existing in the soluble form in human saliva (Fig. 2). Levels of TLR2 stimulants correlated with levels of TLR4 stimulants in both the insoluble fraction ($R^2 = 0.7136$, $p < 0.001$) and the soluble fraction ($R^2 = 0.8501$, $p < 0.001$). There were no significant differences in PAMP concentrations between smokers and non-smokers and there was no correlation between number of sites of pocket depth > 4 mm, or age and PAMP concentrations.

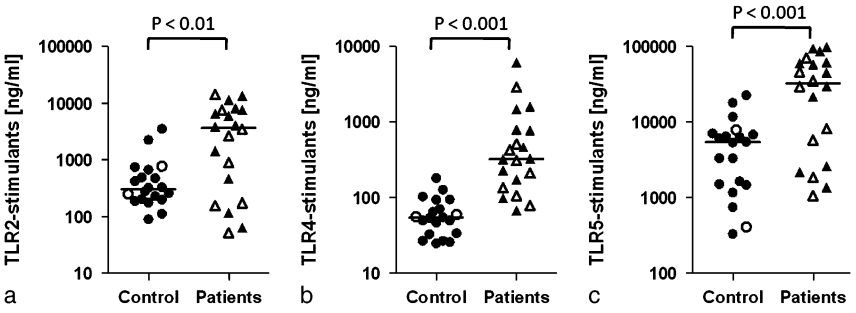

Fig. 1. Quantification of Toll-like receptor (TLR) stimulants in heat-treated saliva. The biological activities of total stimulants of TLR2 (a), TLR4 (b) and TLR5 (c) were quantified relative to Pam_3CSK_4, *Escherichia coli* LPS and *Salmonella typhimurium* flagellin standards, using TLR-transfected HEK-293 cells as described in Materials and methods, in heat-treated saliva from healthy subjects ($n = 20$) and periodontitis patients ($n = 20$). Open symbols represent smokers.

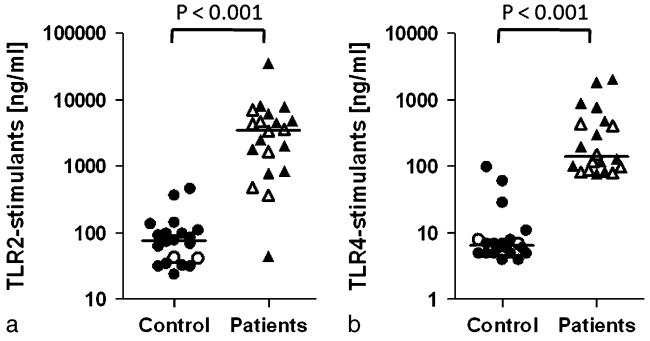

Fig. 2. Quantification of Toll-like receptor (TLR) stimulants in filter-sterilized saliva. The biological activities of soluble stimulants of TLR2 (a) and TLR4 (b) were quantified relative to Pam_3CSK_4 and *Escherichia coli* lipopolysaccharide (LPS) standards, using TLR-transfected HEK-293 cells as described in Materials and methods, in filter-sterilized saliva from healthy subjects ($n = 20$) and periodontitis patients ($n = 20$). Open symbols represent smokers.

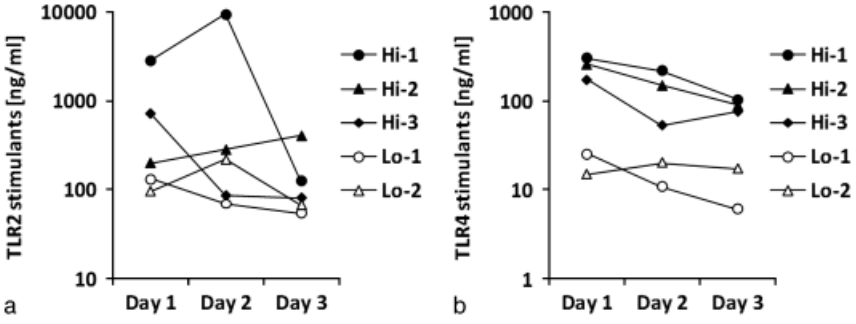

Fig. 3. Daily variation in Toll-like receptor (TLR)2 and TLR4 stimulants in human saliva. The biological activities of soluble stimulants of TLR2 (a) and TLR4 (b) were quantified in filter-sterilized saliva from three healthy subjects with elevated TLR4 stimulants and two healthy subjects with normal levels of TLR4 stimulants on 3 separate days within a 5-day period.

Daily variation in TLR-stimulant concentrations in human saliva

In order to examine the stability of oral PAMP profile with time, three healthy subjects with the highest oral TLR4 stimulants, and two healthy subjects with average levels of oral TLR4 stimulants, were asked to provide further saliva samples on 3 separate days within a 5-day period approximately 2 months after the initial sampling. Median lipopeptide levels of the chosen low and high responders were 29 *versus* 369 ng/ml, respectively, at the first timepoint, and 82 *versus* 281 ng/ml over the 3 days tested 2 months later. Median LPS levels were 7 *versus* 42 ng/ml at the first timepoint, and 16 *versus* 149 ng/ml in the same subjects 2 months later. Oral TLR4-stimulant levels were significantly higher in high responders than in low responders when pooled data from the 3 days examined were compared ($p < 0.001$), indicating that oral TLR4 stimulants may be relatively stable with time (Fig. 3). A similar, although weaker, trend was observed with respect to oral TLR2 stimulants over the 3 days examined ($p = 0.051$).

Molecular characterization of TLR2 and TLR4 stimulants in human saliva

As a variety of molecules of diverse origin and structure have been proposed to stimulate TLR2 or TLR4 (Kumar et al. 2009), we aimed to establish which class of molecule may be responsible for stimulating TLR2 and TLR4 signalling in human saliva. A specific inhibitor of bacterial lipopeptide signalling, OxPAPC, which was shown previously to inhibit lipopeptide signalling but not general downstream TLR- or cytokine-signalling components, significantly blocked TLR2-dependent signalling induced by patient saliva samples (Fig. 4a). Polymyxin-B, an agent that binds and neutralizes LPS, significantly inhibited TLR4 signalling induced by patient saliva samples (Fig. 4b). These data suggest that the majority of the TLR2 and TLR4 stimulants present in human saliva are bacterial lipopeptides and LPSs, respectively.

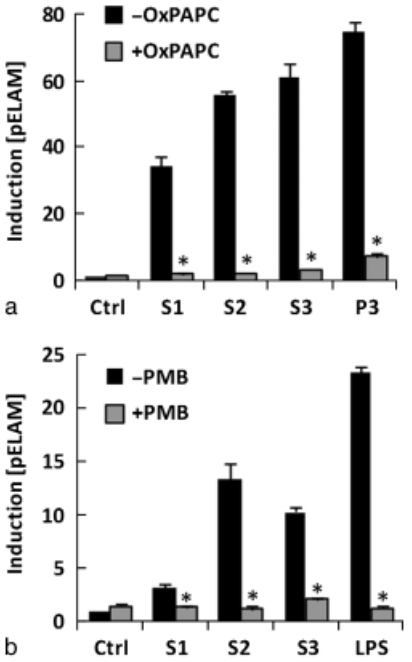

Fig. 4. Effect of lipopeptide and lipopolysaccharide (LPS) inhibitors on oral Toll-like receptor (TLR)-stimulant signalling. Filter-sterilized saliva from three patients was diluted 1:100 in tissue culture medium and applied to HEK-293 cells transfected with TLR2 (a) or TLR4/MD2 (b) in the presence or absence of 25 μg/ml OxPAPC (an inhibitor of bacterial lipopeptide signalling) or 10 μg/ml polymyxin-B (PMB, an inhibitor or LPS signalling). Fold induction of NF-κB sensitive reporter (pELAM) was measured relative to cells cultured in medium alone after 18 h. Positive controls were 10 ng/ml Pam_3CSK_4 (P3) or *Escherichia coli* LPS. $^*p < 0.05$. OxPAPC, oxidized palmitoyl arachidonyl phosphatidyl choline.

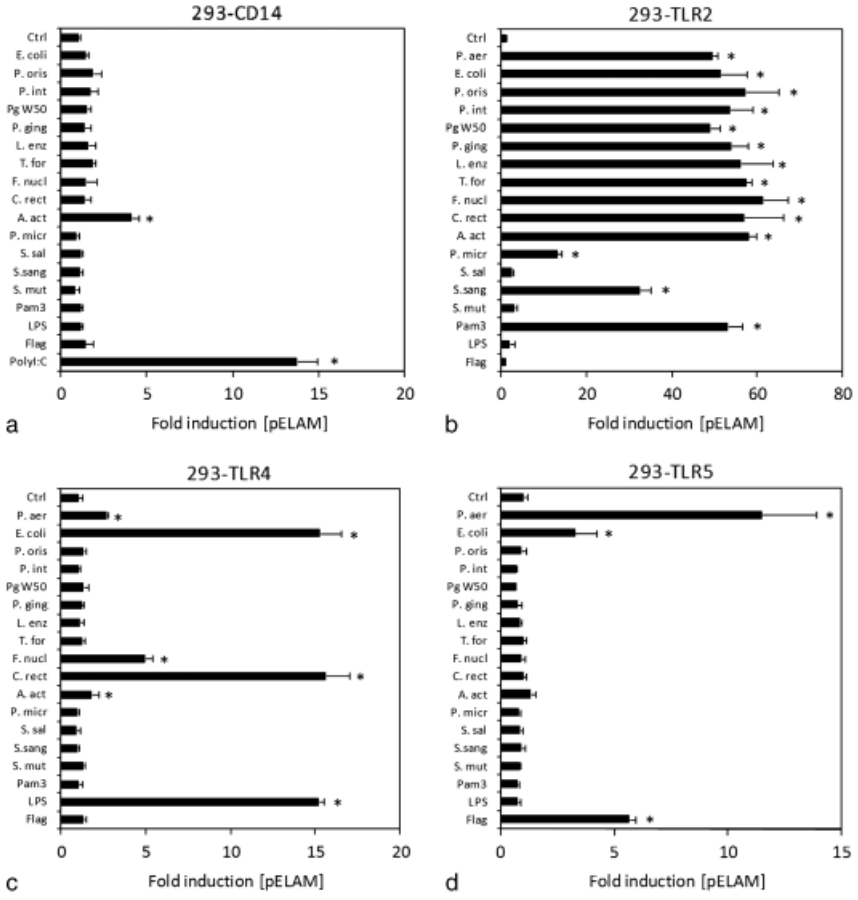

Fig. 5. Capacity of defined oral bacterial isolates to stimulate TLR2, TLR4 and TLR5 signalling. Defined cultures of oral bacteria were heat-killed and applied at a concentration of 10^7 bacteria/ml to HEK-293 cells transfected with CD14 (a), TLR2 (b), TLR4/MD2 (c) or TLR5 (d) and NF-κB-dependent reporter (pELAM). Positive controls for NF-κB activation were 10 ng/ml *Escherichia coli* LPS, 10 ng/ml Pam_3CSK_4 (Pam3), 10 ng/ml *Salmonella typhimurium* flagellin (Flag) or 10 μg/ml polyinosinic acid (PolyI:C). Mean fold induction of NF-κB reporter from triplicate cultures relative to cells cultured in medium alone (Ctrl) is shown $\pm$ SD. Results are representative of at least three experiments.

TLR stimulation by cultured oral bacteria

In order to identify potential bacterial contributors to the pools of TLR stimulants in saliva, we next examined a panel of common oral microorganisms in terms of their potential to stimulate TLR signalling in transfected HEK-293 cells. As expected, HEK-293 cells were insensitive to most bacteria in the absence of TLR co-transfection (Fig. 5a). Surprisingly, however, a modest but significant TLR-independent activation of NF-κB signalling was reproducibly observed in response to *A. actinomycetemcomitans*. Although most of the isolates stimulated TLR2-dependent signalling as expected, several streptococcus species stimulated only a weak or not-detectable TLR2-dependent signal, even when re-examined at higher concentrations (Fig. 5b and data not shown). The Gram-negative oral organisms *C. rectus*, *A. actinomycetemcomitans* and *F. nucleatum* stimulated TLR4-dependent signalling, whereas *T. forsythensis*, *L. enzymogenes*, *P. intermedia*, *P. oris* and two strains of *P. gingivalis* did not (Fig. 5c). TLR5 signalling was induced only by the flagellated organisms *E. coli* and *P. aeruginosa* (Fig. 5d).

Shedding of TLR2 and TLR4 stimulants by *E. coli*

We next aimed to establish the normal range of TLR2 and TLR4 stimulants shed by a model enterobacterial organism, *E. coli* K12, under log-phase growth conditions. Quantification of the soluble PAMPs in growth supernatant from cultured *E. coli* K12 revealed a linear relationship between bacterial cell numbers and concentrations of soluble TLR2 and TLR4 stimulants ($R^2 = 0.867$ and 0.755, respectively, Fig. 6). Calculation of the ratios between lipopeptide or LPS equivalents and bacterial cell concentrations revealed that soluble LPS equivalents averaged 3.1 ng/10^6 bacteria and soluble lipopeptide equivalents averaged 0.7 ng/10^6 bacteria.

Discussion

The present study identified that median levels of soluble TLR2 and TLR4 stimulants were around 80 and 7 ng/ml, respectively, in saliva of healthy subjects whereas soluble TLR2 and TLR4 stimulants were approximately 20- and 50-fold more abundant in saliva of

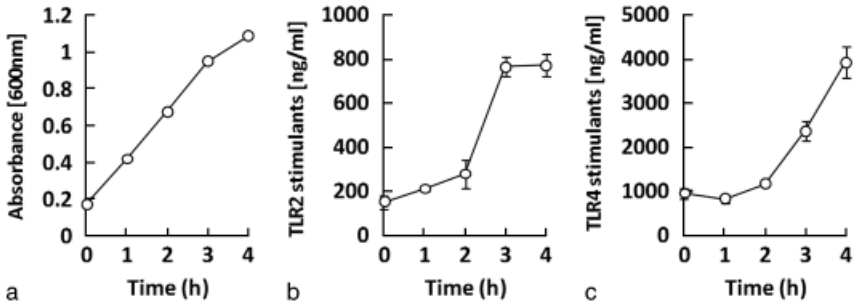

Fig. 6. Quantification of Toll-like receptor (TLR)2 and TLR4 stimulants in log-phase *Escherichia coli* growth medium. *E. coli* K12 cultures grown in Luria broth (LB) at 37°C for 4 h were monitored hourly for absorbance at 600 nm (a), biological activity of soluble TLR2 stimulants in growth medium (b) and biological activity of soluble TLR4 stimulants in growth medium (c). Results shown are means of triplicate measurements ± SD and are representative of atleast three experiments. TLR, Toll-like receptor.

periodontitis patients, respectively (Fig. 2). To our knowledge, only one previous study has measured endotoxin concentrations in saliva of healthy subjects, with normal levels reported to be around 1 μg/ml as measured by the limulus amoebocyte lysate (LAL) assay (Leenstra et al. 1996). However, as our aim was to measure the relative biological activity of TLR stimulants in saliva, we found that the LAL assay was not suitable for this purpose for several reasons. First, the endotoxins of a number of Gram-negative oral organisms have been shown to be antagonists, rather than agonists, of human TLR4 (Yoshimura et al. 2002, Coats et al. 2003, Kikkert et al. 2007), while these endotoxins may stimulate a positive reaction in the limulus assay (Erridge et al. 2007b). Next, as the limulus assay is based upon components of the innate immune system of the horseshoe crab, inter-species receptor differences may lead to an inaccurate estimation of the pro-inflammatory potential of endotoxins in human systems. The overestimation of the endotoxin content of saliva by the LAL assay in earlier studies likely reflects these issues, although our findings confirm the earlier observation that endotoxin levels in saliva tend to remain relatively stable with time in healthy subjects (Leenstra et al. 1996). Finally, the limulus assay is not capable of detecting lipopeptides or flagellins and therefore cannot be used for their quantification (Erridge & Samani 2009). In order to overcome these difficulties, a bioassay-based approach using receptors of the human innate immune system was used to quantify the abundance of TLR stimulants in human saliva.

This approach revealed that stimulants of TLR2, TLR4 and TLR5 are present at significantly higher concentrations in saliva of periodontitis patients than in saliva of healthy subjects. It is possible that these differences could be partly explained by differences in salivary flow rates, which were not measured in this study. However, the magnitude of these differences suggest that other mechanisms are more likely to explain the majority of the differential. Previous studies suggest that the observed differences are also not likely to be due to increased total bacterial load in saliva of periodontitis patients, as total bacterial counts have been reported to be similar in saliva of subjects with gingivitis, periodontitis and good periodontal health (Mantilla Gómez et al. 2001, Mager et al. 2003).

Instead, an alternative explanation may lie in the well-established shift in the oral microflora balance towards Gram-negative organisms in periodontitis (Dzink et al. 1985). We showed recently that Gram-negative organisms generally secrete $\sim$ 100–1000-fold more soluble TLR2 stimulants than Gram-positive organisms (Erridge et al. 2010). Thus, expansion of the sub-gingival Gram-negative microflora may lead to an increased shedding of soluble TLR stimulants in the mouth, which may be detected in saliva. We did not see a correlation between TLR stimulants and mean pocket depth, although this may reflect the fact that mean pocket depths were quite similar in the patient group. Further work will be required to determine if the oral TLR-stimulant profile may be different in untreated periodontitis patients before supportive therapy, and whether elevated oral TLR stimulants serve as markers or as mediators of periodontal disease. Supportive of the latter possibility is the observation that gingival injections of LPS result in inflammation, apical migration of the junctional epithelium and alveolar bone loss in rats (Dumitrescu et al. 2004, Rogers et al. 2007, Tomofuji et al. 2007). Likewise, TLR2 stimulants may also have the potential to promote periodontitis, as it was shown that TLR2-deficient mice are resistant to bone loss in a *P. gingivalis*-mediated model of periodontitis (Burns et al. 2006).

We found that six strains of oral Gram-negative bacteria from a panel of nine examined did not stimulate TLR4-dependent signalling (Fig. 5). These results are consistent with previous reports that endotoxins of several oral Gram-negative organisms do not stimulate signalling via human TLR4/MD2 (Yoshimura et al. 2002, Coats et al. 2003, Kikkert et al. 2007). This apparent evasion of detection by TLR4 was shown to be achieved by modification of the acylation and phosphorylation patterns of the lipid-A expressed by these organisms, such that their structures differ from the hexa-acyl twin phosphate pattern displayed by entereobacterial LPS, which is the optimal stimulant of human TLR4/MD2 (Coats et al. 2003).

It has been proposed that products of the oral microbiota may contribute to the development of atherosclerosis and insulin resistance (Beck et al. 2001, Tonetti 2009, Teeuw et al. 2010). Traditionally, it has been assumed that the mechanisms underlying these observed associations involve transient endotoxaemias and bacteraemias induced by toothbrushing or chewing (Geerts et al. 2002, Pussinen et al. 2004, Lockhart et al. 2008). However, we have recently proposed that bacterial products present in the small intestine may be absorbed with dietary fat to promote low-grade systemic inflammation, thereby potentiating these diseases (Erridge et al. 2007a, Erridge 2008, 2009). As LPS and lipopeptide retain biological activity following protease treatment or low pH (Erridge 2010), it is possible that swallowed products of the oral microflora may survive passage through the stomach to contribute to the biologically active pools of TLR stimulants in the small intestine. The present findings therefore suggest that if healthy subjects swallow approximately 1 l saliva/day,

$\sim 7\,\mu$g LPS and $\sim 80\,\mu$g lipopeptide may also be ingested each day.

By comparison with the oral microflora, the endogenous microflora of the small intestine is relatively limited, being generally $<10^2$ CFU/ml in the duodenum, 10^0–10^4 CFU/ml in the jejunum, 10^3–10^6 CFU/ml in the proximal ileum and 10^5–10^8 CFU/ml in the most distal section of the ileum (Drasar et al. 1969, Posserud et al. 2007). However, Gram-negative organisms are rare in the small intestine and represent only a small fraction of these numbers (Berg 1996, Posserud et al. 2007). Thus, in health, it is likely that enterobacterial species, which we showed recently are likely to represent the major contributors to the endogenous soluble TLR2 or TLR4 stimulants in the small intestine (Erridge et al. 2010), rarely exceed 10^5 organisms/ml. The results presented in Fig. 6 therefore suggest that the maximum concentration of LPS or lipopeptide derived from the resident microflora of the small intestine is likely to be around 0.3 ng/ml LPS and 0.1 ng/ml lipopeptide. These preliminary estimates therefore suggest for the first time that under most conditions the TLR2 and TLR4 stimulants present in the small intestine are likely to derive largely from the oral microflora, rather than from the indigenous microflora of the small intestine. Further studies involving direct sampling of contents of the terminal ileum will be required to examine this hypothesis further.

In summary, we report for the first time that periodontal disease is associated with marked increases in salivary concentrations of stimulants of TLR2 and TLR4, relative to healthy subjects. While the relevance of the present findings are yet to be elucidated, the accumulating evidence is that periodontitis may increase the risk of developing diseases such as atherosclerosis and insulin resistance via mechanisms that involve chronic inflammatory signalling pathways. Further research will be required to determine if elevated salivary PAMP concentrations may serve as markers or as mediators of periodontitis and these associated diseases.

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Clinical Relevance

Scientific rationale for the study: The mechanisms underlying the observed associations between periodontitis, atherosclerosis and insulin resistance are currently unclear.

Principal findings: We show that concentrations of stimulants of TLR2 and TLR4, which have been proposed to promote insulin resistance and atherosclerosis in animal models and human epidemiological studies, are significantly higher in saliva of patients with periodontitis than in saliva of healthy subjects.

Practical implications: These data identify two novel and readily accessible biomarkers of periodontal disease, and support the emerging hypothesis that oral TLR stimulants may contribute to the observed associations between periodontal disease and cardiovascular and metabolic risk.