Activation of Toll-like receptor 2 induces B₁ and B₂ kinin receptors in human gingival fibroblasts and in mouse gingiva

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The regulation of the kallikrein-kinin system is an important mechanism controlling vasodilation and promoting inflammation. We aimed to investigate the role of Toll-like receptor 2 (TLR2) in regulating kinin B₁ and B₂ receptor expression in human gingival fibroblasts and in mouse gingiva. Both P. gingivalis LPS and the synthetic TLR2 agonist Pam₃CSK₄ increased kinin receptor transcripts. Silencing of TLR2, but not of TLR4, inhibited the induction of kinin receptor transcripts by both P. gingivalis LPS and Pam₃CSK₄. Human gingival fibroblasts (HGF) exposed to Pam₃CSK₄ increased binding sites for bradykinin (BK, B₂ receptor agonist) and des-Arg¹⁰-Lys-bradykinin (DALBK, B₁ receptor agonist). Pre-treatment of HGF for 24 h with Pam₃CSK₄ resulted in increased PGE₂ release in response to BK and DALBK. The increase of B₁ and B₂ receptor transcripts by P. gingivalis LPS was not blocked by IL-1β neutralizing antibody; TNF-α blocking antibody did not affect B₁ receptor up-regulation, but partially blocked increase of B₂ receptor mRNA. Injection of P. gingivalis LPS in mouse gingiva induced an increase of B₁ and B₂ receptor mRNA. These data show that activation of TLR2 in human gingival fibroblasts as well as in mouse gingival tissue leads to increase of B₁ and B₂ receptor mRNA and protein.

Kinins are generated by the release from kininogens through the enzymatic action of kallikreins. Since their discovery, these peptides are well known as pro-inflammatory molecules by increasing vasodilation, vascular permeability and cellular migration. The kinin family is composed of bradykinin (BK) and Lys-bradykinin (Lys-BK), both B₂ receptor agonists, and des-Arg¹⁰-Lys-bradykinin (DABK) and des-Arg¹¹-Lys-bradykinin (DALBK), B₁ receptor agonists. B₂ receptors are constitutively expressed in many cell types and are responsible for the classical actions of kinins, while B₁ receptors are induced under pathological conditions and are mainly involved in inflammatory events. Mechanisms controlling the local actions of the kallikrein-kinin system involve release of kinins but also regulation of their receptors. Thus, pro-inflammatory molecules such as cytokines and lipopolysaccharide (LPS) regulate B₁ and B₂ receptor expression.

Periodontal disease is a highly prevalent chronic inflammatory disease of the periodontium causing loss of gingival tissue, periodontal ligament and tooth-supporting bone. Colonization of the root surfaces on teeth by complex subgingival biofilms, containing several gram-negative bacteria, including Porphyromonas gingivalis, initiates the cascade of a wide variety of events leading to infiltration of inflammatory cells and the production of molecules that can disturb the remodeling of periodontal tissues, eventually leading to loss of alveolar bone and loosened teeth. The presence of P. gingivalis impedes or modulates the host protective mechanisms in many different ways and is associated with diseased sites. Therefore, P. gingivalis is potentially a keystone pathogen that modifies the environment supporting the bacterial community to promote periodontal disease.

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We have reported that kinins may play important roles in periodontitis\(^7\). Accordingly, B\(_1\) and B\(_2\) receptors are expressed on osteoblasts and fibroblasts and activation of these receptors causes enhanced bone resorption mediated by increased prostaglandin E\(_2\) (PGE\(_2\)) formation in both cell types and enhanced expression of receptor activator of nuclear factor-\(\kappa\)B ligand (RANKL) in osteoblasts\(^8,9\). Interestingly, \(P.\ gingivalis\) expresses an arginine specific cysteine proteinase (Arg-gingipain-1/RGP-1) that can release kinins from kininogens\(^10\), facilitated by components of the kallikrein-kinin system binding to gingipains on the cell surface of \(P.\ gingivalis\)\(^11\).

Toll-like receptors are a family of pattern recognition receptors that recognize a plethora of pathogen-associated molecular patterns (PAMPs). To the PAMPs belongs lipopolysaccharide (LPS) from gram-negative bacteria, which is recognized by Toll-like receptors 4 (TLR4)\(^12\). The importance of TLR4 for periodontal disease is well studied, but much less is known on the role of TLR2. Interestingly, \(P.\ gingivalis\) has the capacity to activate both TLR2 and TLR4\(^13,14\). Recently, we reported that \(P.\ gingivalis\) stimulates osteoclast formation \(in\ vitro\) and causes inflammation induced bone loss \(in\ vivo\) through activation of TLR2\(^15\). This observation and the fact that periodontitis induced by \(P.\ gingivalis\) can not be observed in mice with genetic deletion of TLR2 indicates that TLR2 is also important for the pathogenic properties of \(P.\ gingivalis\) in periodontal disease\(^16-18\).

Data from human and mouse studies have evidenced an association between periodontal disease and rheumatoid arthritis (RA)\(^19-21\). The observation that alveolar bone loss in periodontitis patients precede the clinical onset of symptoms of RA\(^21\), together with the fact that treatment of periodontitis seems to reduce the severity of RA\(^22,23\) indicates a possible cause relationship between the two diseases. Further support for a role of oral infection in RA are studies in mice showing that oral infection with \(P.\ gingivalis\) aggravates arthritic bone erosions in collagen-induced arthritis\(^22,24\). The pathogenetic mechanisms involved were, at least in part, dependent on Th17 cells through the activation of TLR2 by \(P.\ gingivalis\)\(^24\). Further supporting an association between periodontal disease and RA is the observation that DNA from \(P.\ gingivalis\) has been detected in serum and synovial fluid from RA patients\(^25\). The routes used by \(P.\ gingivalis\) to invade blood vessels in the periodontium and to reach the joints through the circulation are still unknown, but may be attributed to local activation in the periodontal tissues of the kallikrein-kinin system. This hypothesis is supported by the fact that local vascular permeability and bacterial spreading can be enhanced by \(P.\ gingivalis\) through a mechanism that was inhibited by decreasing kinin activity, either by administration of angiotensin converting enzyme (ACE), acting as a kininase enzyme, or by a kinin B2 receptor antagonist. In contrast, increased kinin activity by administration of BK, or the ACE inhibitor captopril, enhanced vascular permeability and bacterial spreading induced by infection with \(P.\ gingivalis\)\(^26\). Interestingly, the ability of \(P.\ gingivalis\) to disseminate was strain specific and correlated to generation of kinin activity. Thus, local regulation of kinin receptors in gingival fibroblasts could contribute by increasing the response to BK, leading to the generation of vasoactive mediators, such as prostaglandins, and by promoting bacterial spreading and aggravation of RA in periodontitis patients. In the present study, we have investigated the role of TLR2 for the local regulation of kinin receptors and report the novel finding that activation of TLR2 directly increases the expression of functional B\(_1\) and B\(_2\) receptors in human gingival fibroblasts as well as in mouse gingival tissue.

**Results**

**Induction of BDKRB1 and BDKRB2 mRNA expression by \(P.\ gingivalis\) LPS and Pam\(_2\)CSK\(_4\) in HGF.** Human gingival fibroblasts were isolated from an individual without any clinical signs of gingival inflammation. Exposure of these cells to \(P.\ gingivalis\) LPS (10 \(\mu\)g/ml) for 3–24 hours resulted in time-dependent increased expression of both BDKRB1 (Fig. 1A) and BDKRB2 mRNA (Fig. 1B). The upregulation of BDKRB1 and BDKRB2 mRNA caused by \(P.\ gingivalis\) LPS was concentration dependent, with stimulatory effects seen at and above 100 ng/ml (Fig. 1C,D). Expression of IL6 mRNA has previously been reported to be upregulated by \(P.\ gingivalis\) LPS\(^27\). In the present experiments, increased IL6 mRNA was seen at the same concentrations as those stimulating kinin receptor expression (data not shown). BDKRB1 and BDKRB2 mRNA expression was enhanced also by the synthetic TLR2 agonist Pam\(_2\)CSK\(_4\) (50 ng/ml) (Fig. 1E).

In order to evaluate if regulation of kinin receptor expression by \(P.\ gingivalis\) LPS and Pam\(_2\)CSK\(_4\) in gingival fibroblasts was a general phenomenon, we incubated cells isolated from five different individuals with these test substances. In cells from all five individuals, Pam\(_2\)CSK\(_4\) (50 ng/ml) significantly increased both BDKRB1 and BDKRB2 mRNA expression (Fig. 2). \(P.\ gingivalis\) LPS (1 \(\mu\)g/ml) significantly increased both BDKRB1 and BDKRB2 mRNA expression in cells from four of the five patients (Fig. 2).

**Induction of Bdkrb1 and Bdkrb2 mRNA expression by \(P.\ gingivalis\) LPS in mouse gingiva.** In order to assess if the upregulation of BK receptors by \(P.\ gingivalis\) LPS observed in the fibroblasts cultures could be observed also \(in\ vivo\), we locally exposed gingival tissue in mice to the bacterial LPS. Injection of \(P.\ gingivalis\) LPS (3 \(\mu\)g) every other day for 14 days in mouse gingiva enhanced the mRNA expression of Bdkrb1 and Bdkrb2 (Fig. 3A,B). Bdkrb1 mRNA was increased by 2.3-fold (Fig. 3A), while Bdkrb2 mRNA was increased by 1.6-fold (Fig. 3B).

**LPS from \(P.\ gingivalis\) and Pam\(_2\)CSK\(_4\)** up-regulate kinin receptor transcripts selectively via TLR2. In order to confirm that up-regulation of kinin receptors by the TLR2 receptor agonists used was a specific effect of TLR2 receptor activation, we knocked down TLR2 by using small interfering RNA designed to silence TLR2 (TLR2-siRNA). To rule out the contribution of TLR4, we also silenced TLR4 using TLR4-siRNA. The mRNA expression levels of TLR2 and TLR4 were decreased by 90%, as compared to cells transfected with a control (scrambled) siRNA (SCR-siRNA; data not shown). Our results showed that knockdown of TLR2 significantly decreased the enhancement of BDKRB1 and BDKRB2 mRNA induced by \(P.\ gingivalis\) LPS, as well as by Pam\(_2\)CSK\(_4\) (Fig. 4A,B). In contrast, knockdown of TLR4 did not significantly affect kinin receptor expression induced by \(P.\ gingivalis\) LPS or by Pam\(_2\)CSK\(_4\) (Fig. 4C,D).
TLR2 agonists up-regulate kinin receptors at protein level. As shown in Fig. 5A,B, gingival fibroblasts pre-treated with Pam3CSK4 for 24 h exhibited enhanced binding to [3H]-BK and [3H]-DALBK, evidencing that the number of correctly folded receptor proteins capable of binding to the kinin receptors was enhanced.

To analyze the functional relevance of the up-regulation of B1 and B2, we took advantage of the fact that activation of both receptors are linked to increased formation of PGE2 in gingival fibroblasts 28,29, as well as in many other cell types. When the fibroblasts were pre-treated with Pam3CSK4 for 6 h (50 ng/mL), the subsequent PGE2 responses to both BK and DALBK were enhanced (Fig. 5C), indicating that activation of TLR2 results in increased number of functional kinin receptors.

Analysis of the participation of IL-1β and TNF-α in P. gingivalis LPS-induced kinin receptor expression. In the human gingival fibroblasts, LPS from P. gingivalis induced the expression of IL-1β and TNF-α mRNAs, which were undetectable in control cells not exposed to LPS (data not shown). We, therefore, evaluated if these cytokines could participate in kinin receptor expression induced P. gingivalis LPS and for these purpose made use of specific neutralizing antibodies tested to verify their effectiveness (Supplemental 2). Neither the antibody neutralizing IL-1β (Fig. 6A), nor the one neutralizing TNF-α (Fig. 6C), affected P. gingivalis LPS (1 μg/mL)-induced increase of the mRNA expression of BDKRB1. At variance, although the treatment with
the IL-1β neutralizing antibody caused no effect on BDKRB2 mRNA induced by P. gingivalis (Fig. 6B), the TNF-α neutralizing antibody partially inhibited the up-regulation of BDKRB2 mRNA (Fig. 6D).

**Discussion**

In the present study, we report that the mRNAs encoding for the B₁ and B₂ kinin receptors are among those genes regulated by LPS from the periodontopathogenic bacterium P. gingivalis, both in vitro in human gingival fibroblasts and in vivo in mouse gingiva. Interestingly, it has been demonstrated that expression of TLR2 mRNA and protein, one of the receptors activated by P. gingivalis is enhanced by activation of B₂ kinin receptor, indicating a bidirectional regulation of kinin receptors and TLR2 by their cognate ligands. In order to escape from the host recognition by the innate immune system and promote its adaptive fitness in the mammalian host, P. gingivalis LPS may elicit different responses when bound to TLR2 or TLR4. The heterogeneous responses of P. gingivalis LPS observed in vitro and in vivo may be due to the fact that many preparations are contaminated with lipoproteins or other lipid species. Although TLRs are mainly present in inflammatory cells, it has been shown that gingival fibroblasts express a number of proteins belonging to the
Noteworthy, in the present study we show that human gingival fibroblasts are capable of up-regulating this receptor by cardiac myocytes challenged with LPS was partially dependent on TNF-α and TNF-β. As regards the up-regulation, it has previously been shown that the up-regulation of B1 and B2 receptors, independently of IL-1β, may be of importance for the actions of kinins in the periodontium in chronic inflammation. Underlying the invasion of periodontogenic bacteria is still elusive, but one possible route could be local activation of the kallikrein-kinin system from a sequestered infection site to promote vasodilation and facilitate invasion.

We have previously reported that IL-1β and TNF-α enhance the expression of BDKRB1 and BDKRB2 in human gingival fibroblasts. Since induction of pro-inflammatory cytokines is a well-recognized response to TLR2 activation, we investigated if these cytokines mediated the effects by using antibodies that specifically neutralize the effects of IL-1α and TNF-α, we show that up-regulation of BDKRB1 occurs independently of the production of both cytokines, whereas BDKRB2 up-regulation is partially dependent on TNF-α production but independent on IL-1β. In the mouse paw model, where inflammatory cells can be recruited to the inflamed site, neutrophil influx and TNF-α production are important events for the regulation of BDKRB1 levels by P. gingivalis LPS. Although TNF-α expressing neutrophils are present in the inflamed gingiva during periodontitis, the up-regulation of BDKRB1 in gingival fibroblasts, independently of IL-1β and TNF-α, may be of importance for the actions of kinins in the periodontium in chronic inflammation. As regards the BDKRB2 up-regulation, it has previously been shown that the up-regulation of this receptor by cardiac myocytes challenged with LPS was partially dependent on TNF-α production, in agreement with our data. Nevertheless, it is interesting to note that the BDKRB2 up-regulation by P. gingivalis LPS was not completely inhibited by TNF-α neutralizing antibody in the gingival fibroblasts, which means that other TNF-α-independent pathways may also be involved in the regulation of the expression of this receptor.

The data presented here may be of clinical relevance, since activation of TLR2 by P. gingivalis is associated with the aggravation of experimental arthritis in mice. In humans, an association between periodontitis and RA has been demonstrated. One possible mechanism has been suggested to be due to the presence of PAMPs derived from oral bacteria in the diseased joints. Supporting this view, DNA from periodontopathogenic bacteria can be detected in the serum and synovial fluid from patients with RA and psoriatic arthritis. The mechanism underlying the invasion of periodontogenic bacteria is still elusive, but one possible route could be local activation of the kallikrein-kinin system from a sequestered infection site to promote vasodilation and facilitate invasion.

Our data can be reconciled with this hypothesis, since activation of TLR2 by P. gingivalis LPS not only increased the expression of Bdkrb1 and Bdkrb2 in mouse gingiva. Injection of LPS from P. gingivalis (3 μg) every other day for 14 days increases the expression of Bdkrb1 (A) and Bdkrb2 (B) in mouse gingiva in comparison with injection of vehicle (Control). The expression was analysed using Taqman assays. Data were normalized against Actb and are expressed as percent of the means for the controls, which was arbitrarily set to 100%. Each symbol represents data from one mouse. The horizontal line represents the mean for each experimental group. ** and *** indicate significant difference to untreated mice, P < 0.01 and P < 0.001, respectively. Statistical analysis was determined using Student’s unpaired t-test.

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**Figure 3.** *P. gingivalis* LPS increases the expression of Bdkrb1 and Bdkrb2 in mouse gingiva. Injection of LPS from *P. gingivalis* (3 μg) every other day for 14 days increases the expression of Bdkrb1 (A) and Bdkrb2 (B) in mouse gingiva in comparison with injection of vehicle (Control). The expression was analysed using Taqman assays. Data were normalized against Actb and are expressed as percent of the means for the controls, which was arbitrarily set to 100%. Each symbol represents data from one mouse. The horizontal line represents the mean for each experimental group. ** and *** indicate significant difference to untreated mice, P < 0.01 and P < 0.001, respectively. Statistical analysis was determined using Student’s unpaired t-test.
B1 and B2 receptors mRNA, but also increased the capacity of gingival fibroblasts to produce prostaglandin E2, a potent vasodilator agent, in response to the kinins. Kinins themselves are also vasodilatory agents, and can be generated at the inflammatory site during periodontal infection by the action of gingipain, a kinin-producing protease expressed by *P. gingivalis*. The proposed sequence of events involved in bacterial invasion promoted by *P. gingivalis*, including a role of TLR2 induced kinin expression in fibroblasts, is outlined in Fig. 7.

In conclusion, in this study we report that *P. gingivalis* LPS is able to up-regulate kinin receptors in human gingival fibroblasts and mouse gingiva by the activation of TLR2. Moreover, our data reveal a new pathway by which these receptors are up-regulated which is independent on the production of IL-1β and TNF-α in the case of B₁ receptor, and partially dependent on TNF-α production in the case of B₂ receptor. These findings open new horizons for studies investigating mechanisms controlling the expression of B₁ and B₂ receptors in non-inflammatory cells.

**Material and Methods**

**Materials.** Specified in Supplementary Material 1.

**Cell culture.** Human gingival fibroblasts were isolated from healthy donors with written, informed consent as previously described. Fibroblasts were from different individuals (males and females between 25–50 years of age (all generally and periodontally healthy) and the cells used in the present study were from passage 5–10. Approval from the Ethical Committee for Human Research at Umeå University was obtained for all the methods described, and all methods were performed in accordance with the relevant guidelines and regulations. The data shown were obtained using cells from one individual but reproduced using cells from another individual with the exception of the experiments used to produce data shown in Fig. 2 which were performed using cells from five different individuals.
In vivo regulation of Bdkrb1 and Bdkrb2 by P. gingivalis LPS. In order to assess the effect of P. gingivalis LPS on the regulation of kinin receptors in vivo, we injected LPS from P. gingivalis (3 μg per injection), or PBS, in the gingiva in the mesial aspect of upper first molar of male 6-weeks old C57Bl/6 mice. The protocol for this experiment was approved by the Ethical Committee on Animal Experimentation at the School of Dentistry.
in Araraquara – UNESP, Brazil and performed in accordance with the guidelines from the Brazilian College for Animal Experimentation (COBEA). Injections were performed every second day for 14 days, and the animals were sacrificed 6 h after the last injection. The gingival tissue was dissected and the RNA was extracted using RNAqueous-MICRO kit for qPCR analysis.

**RNA extraction and cDNA synthesis.** After exposure to the test substances for the time indicated in the graphs or figure legends, total RNA was extracted from the cells using RNAqueous-4PCR kit. The cDNA was synthesized with a first-strand cDNA synthesis kit using oligo(dT)15 as primers following the manufacturer’s instructions.

**Quantitative real-time polymerase chain reaction (qPCR).** The mRNA expression of human BDKRB1, BDKRB2, TLR2, TLR4 and the mouse genes Bdkrb1 and Bdkrb2 were assessed using previously described primer sequences. Amplification was performed in an ABI Prism 7900HT sequence detection system using cDNA as template, specific primers and probes and Taqman Universal Mamer Mix kit. To control the amount of cDNA input, ribosomal protein L13A (RPL13A) or β-actin (Actb) were used as controls (housekeeping genes) for human and mouse samples, respectively.

**Radioligand binding assays.** After overnight attachment of the fibroblasts, the media were changed and α-MEM with 1% FCS with or without Pam3CSK4 (50 ng/mL) was added. Twenty-four hours later, binding studies were performed following all the standardizations described previously. To assess the amount of binding sites, the cells were incubated in MEM/HEPES/0.1% BSA with [3H]-BK 4 nmol/l or [3H]-des-Arg10-Lys-BK 14 nmol/l for 90 min at 4 °C. After extensive washing steps, the cells were detached and the radioactivity analyzed using
The binding of $[^3H]$-BK was competed for by B2, but not B1, ligands and the binding of $[^3H]$-des-Arg$^{10}$-Lys-BK was competed for by B1, but not B2, ligands (data not shown).

**Prostaglandin E$_2$ production.** The amount of PGE$_2$ was measured in the supernatant of cells exposed to BK (1 μM) or DALBK (1 μM) for 24 hours by using a commercially available ELISA kit for PGE$_2$. In order to analyze the effect of TLR2 activation on kinin receptors expression, cells were pre-treated with or without Pam$_2$CSK$_4$ (50 ng/mL) for 24 h prior to the addition of kinins.

**TLR2 and TLR4 knockdown.** TLR2 and TLR4 were knocked down in gingival fibroblasts using siRNA as previously described$^{27}$. Briefly, the cells were transfected with 30 nM of scrambled (SCR – Ambion, AM4635), TLR2 (Ambion, ID#111285) or TLR4 siRNA (Ambion, ID#112337) using lipofectamin 2000 in α-MEM with 10% FCS without antibiotics. The knockdown was confirmed by qPCR and more than 90% inhibition of TLR2 and TLR4 mRNA was achieved (data not shown). Twenty-four hours after transfection, the media were changed and the cells were exposed to the test substances; 6 h later RNA was extracted for qPCR analysis.

**Participation of IL-1β and TNF-α in up-regulation of kinin receptors induced by P. gingivalis LPS.** After overnight attachment, human gingival fibroblasts were incubated in α-MEM/1% FCS in the presence or absence of P. gingivalis LPS, with or without antibodies neutralizing human IL-1β or human TNF-α. The IL-1β neutralizing antibodies blocked IL-1β induced enhancement of BDKRB1 mRNA expression and the TNF-α neutralizing antibodies blocked TNF-α induced increase of BDKRB1 mRNA (Supplementary Material 2).

**Statistical analyses.** Statistical analysis of multiple treatment groups was performed using analysis of variance (ANOVA), with Levene’s homogeneity test, and Dunnett’s T3 or Tukey post hoc test. For the experiments with two groups, the unpaired Student’s t-test was performed. The data shown in the figures are expressed as means ± standard error of means (SEM) for 3–6 wells per experimental group.
Data Availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Author Contributions**
P.P.C.S. and U.H.L. designed the study; P.P.C.S., I.L. and F.A.C.M. conducted the experiments; P.P.C.S., P.L., C.M.C. and U.H.L. interpreted the data; P.P.C.S. and U.H.L. wrote the first draft of the manuscript which was edited and approved by all authors.

**Additional Information**

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