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Regulation of protease-activated receptor-2 expression in gingival fibroblasts and Jurkat T-cells by *Porphyromonas gingivalis*

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**Short title:** PAR-2 activation in GF and T-cells by *P. gingivalis*

**Key words:** *Porphyromonas gingivalis*, PAR-2, gingipains, signaling, inflammation, periodontal disease
Abstract

Periodontal disease destroys the tooth-supporting tissues as a result of chronic inflammation elicited by bacterial accumulation on tooth surfaces. *Porphyromonas gingivalis* is a major periodontal pathogen, with a significant capacity to perturb connective tissue homeostasis and immune responses in the periodontium, attributed to its virulence factors, including a group of secreted cysteine proteases (gingipains). Protease-activated receptor (PAR)-2 is a G-protein-coupled receptor activated upon proteolytic cleavage, mediating intracellular signaling events related to infection and inflammation, such as cytokine production. Gingival fibroblasts (GF) and T-cells have central roles in periodontal inflammation, which can potentially be mediated by PAR-2. The aims of this study were to investigate the effects of *P. gingivalis* on PAR-2 gene expression in human GF and Jurkat T-cells, using quantitative real-time PCR, and to evaluate the involvement of gingipains. After 6 h of challenge with ascending concentrations of *P. gingivalis*, PAR-2 expression was up-regulated in both cell types by approximately 5-fold, compared to the control. The *P. gingivalis* concentration required for maximal PAR-2 induction was 4-fold greater in GF than Jurkat T-cells. Heat-inactivation or chemical inhibition of cysteine proteases abolished the capacity of *P. gingivalis* to induce PAR-2 expression in Jurkat T-cells. In conclusion, *P. gingivalis* can induce PAR-2 expression in GF and Jurkat T-cells, potentially attributed to its gingipains. These findings denote that *P. gingivalis* may perturb the host immune and inflammatory responses by enhancing PAR-2 expression, thus contributing to the pathogenesis of periodontal disease.
1. Introduction

Periodontitis is an infectious disease that destroys the tooth-supporting (periodontal) tissues as a result of chronic inflammation caused by bacterial accumulation on tooth surfaces. It is relatively common in the human population and if left untreated, it may result in tooth loss and subsequently impaired function of mastication and poor aesthetics. The bacterial challenge triggers several complex responses in the resident periodontal tissues, as well as the local cells of the immune system, which can be highly variable among individuals (Schenkein, 2006). The equilibrium between bacteria and host response is the determinant factor, as its disturbance can eventually lead to the destruction of the periodontal connective tissue and supporting alveolar bone, culminating in tooth loss. The host response essentially manifests as a series of inflammatory events that can lead to periodontitis, if excessive (Kornman et al., 1997, Schenkein, 2006). On the histopathological level, the inflammatory responses involve both structural cells of the periodontium, such as the gingival fibroblasts (GF), as well as local cells of the immune system, such as T-cells (Page and Schroeder, 1976). It is now well established that T-cells are a major cell population implicated in the pathogenesis of periodontal diseases, particularly mediating alveolar bone destruction (Taubman and Kawai, 2001).

*Porphyromonas gingivalis* is a gram-negative black pigmenting anaerobe, reported to be a major member of the pathogenic microbiota, particularly in chronic periodontitis (Zambon et al., 1981, Slots et al., 1986, Holt et al., 1999, Yilmaz, 2008). Despite the profound inflammatory response, the host immune system is usually not able to eliminate *P. gingivalis* infection. This bacterial species may therefore have the potential to perturb the normal immune and inflammatory host responses, permitting its colonization and propagation in the oral cavity (Lamont and Jenkinson, 1998). These capacities are attributed to its virulence factors, including its lipopolysaccharide (LPS) (Bainbridge et al., 2002) and gingipains (Curtis et al., 2001). The gingipains in particular are a group of cysteine protease enzymes, believed to be main endopeptidases produced by the bacterium (Kesavalu et al., 1997), as they represent the majority of the outer surface protein on *P. gingivalis* W50 (Veith et al., 2002). These are mainly the Arg-X and the Lys-X gingipains (Curtis et al., 2001). Clinical studies show that *P. gingivalis* infection leads to elevated serum IgG antibody response to the gingipains, in as many as 92% of patients with chronic periodontitis (O’Brien-Simpson et al., 2000). Due to their enzymatic activity, they are considered major virulence factors for periodontal tissue matrix degradation and evasion of host defences. They are reported to degrade the CD14 (Sugawara et al., 2000) and TLR (Kishimoto et al., 2006) receptors on monocytes, as well as pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6 and IL-8 (Calkins et al., 1998, Banbula et al., 1999, Baba et al., 2002, Kobayashi-Sakamoto et al., 2003), immunoglobulins (Gregory et al., 1992), and plasma host protease inhibitors (Grenier, 1996).

Protease-activated receptors (PARs) are a family of G-protein-coupled receptors that mediate a cascade of intracellular signaling events related to injury, infection, inflammation and cell death (Coelho et al., 2003, Ossovskaya and Bunnett, 2004). Their activation depends on proteolytic cleavage of the extracellular N-terminal domain, resulting in the generation of a new “tethered ligand” N-terminal, which can autoactivate the cell. Up to date, four members of the PAR family have been cloned. PAR-1, PAR-3 and PAR-4 can be activated by thrombin, whereas PAR-2 can be activated by several proteases, including cysteine proteases (Lourbakos et al., 1998, Abraham et al., 2000, Vergnolle et al., 2001b). PAR-2 is expressed by virtually all cell types, including epithelial and endothelial cells, fibroblasts, osteoblasts, neutrophils, myocytes, neurons, T-cells and astrocytes (Nystedt et al., 1996, Abraham
et al., 2000, Uehara et al., 2002, Uehara et al., 2003). It is involved in several pathophysiological processes, such as leukocyte migration, cytokine production, inflammation of the joints, skin and kidney, hyperalgesia, allergic inflammation of airways, and periodontal disease (Vergnolle et al., 2001a, Ossovskaya and Bunnett, 2004, Holzhausen et al., 2005b).

It is hypothesised that \textit{P. gingivalis} challenge would induce PAR-2 expression in gingival connective tissue cells, as well as cells of the immune system. Since PAR-2 can be activated by cysteine proteases, it is also postulated that \textit{P. gingivalis} gingipains could be involved in the induction of PAR-2 expression. Therefore, the primary aim of the present \textit{in vitro} study was to investigate the effects of \textit{P. gingivalis} challenge on PAR-2 gene expression in human GF and a human T-cell line. A further aim was to investigate if gingipains are involved the regulation of PAR-2 expression by \textit{P. gingivalis}.

2. Materials and Methods

2.1 Human GF cell cultures

Human GF cell lines were established as previously described (Belibasakis et al., 2005, Belibasakis et al., 2007). Briefly, the gingival tissue biopsies used were obtained from four healthy young individuals (ages, 13 to 16 years), who were about to have their first premolars removed in the course of orthodontic treatment. Ethical approval was granted by the Human Studies Ethical Committee of Umeå University, Sweden, and informed consent was given by all subjects. The obtained cell lines were cultured in Minimum Essential Medium (MEM) Alpha (Gibco BRL Life Technologies, UK), supplemented with 10% heat-inactivated foetal bovine serum (Bio-Whittaker, Maryland, USA), 50 U/ml penicillin, and 50 µg/ml streptomycin (Gibco BRL Life Technologies, UK). All cell lines were confirmed to be free of mycoplasma infections by DAPI fluorescence staining. For the experiments, GF cultures cells at passages 3 or 4, were seeded at concentration $2 \times 10^3$ cells/cm$^2$, and allowed to attach for 24 h, maintaining a sub-confluent status. Thereafter, the cells were cultured for 6 h in presence or absence of \textit{P. gingivalis} bacterial culture supernatants at sub-toxic concentrations, as previously defined (Belibasakis et al., 2007).

2.2 Jurkat T-cell cultures

The Jurkat T-lymphocyte leukemia cell line (E6-1; American Type Tissue Culture Collection) was cultured as previously described (Bostanci et al., 2009). Briefly, the cells were maintained in RPMI Glutamax culture medium (Gibco BRL Life Technologies, UK), which was supplemented with 10% FBS. The cells were harvested and plated in 12-well tissue culture plates, at density $10^6$ cells/well, in 1 ml culture media. Thereafter, the cells were cultured for 6 h in presence or absence of \textit{P. gingivalis} bacterial culture supernatants at sub-toxic concentrations, as previously defined (Bostanci et al., 2009).

2.3 Bacteria and growth conditions

\textit{Porphyromonas gingivalis} W50 wild-type strain expressing both Arg-X gingipain A and Arg-X gingipain B (rgpA rgpB), as well as Lys-X gingipain (kgp), was used in this study. This bacterial strain was cultured in blood agar base supplemented with 5% horse blood (Oxoid, Hampshire, UK) and maintained by weekly subculture for up to 5

3
weeks. Liquid cultures were prepared by inoculation of bacterial colonies (3-4 days old) from blood agar plates into 10 ml brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) supplemented with 5 mg/liter haemin (Sigma, Dorset, UK), and incubated for 24 h. A 10% inoculum was transferred to 90 ml of the same medium and incubated for 6 days. All cultures were grown at 37°C in a Don Whitley anaerobic cabinet, MACS MG500, in an atmosphere of 80% N\textsubscript{2}, 10% H\textsubscript{2}, and 10% CO\textsubscript{2}. After this culture period, bacteria were harvested by centrifugation at 10,000 x g for 15 min at 4°C and the supernatants were collected, filter-sterilized over a 0.2 µm filter, and stored at -80°C until further use. For the experiments, this \textit{P. gingivalis} preparation was diluted in the cell culture medium and its concentration was expressed as total bacterial protein (µg/ml) present in the cell cultures. Protein concentration was determined by Bio-Rad Protein assay (Bio-Rad, Hertfordshire, UK) according to the manufacturer’s instructions.

\section*{2.4 Inhibition of cysteine protease activity in \textit{P. gingivalis}}

To investigate the role of \textit{P. gingivalis} cysteine proteases (gingipains) in PAR-2 mRNA expression by Jurkat T-cells, \textit{P. gingivalis} W50 culture supernatant was pre-treated with 1 mM of the cysteine protease inhibitor Na-p-tosyl-lysine chloromethyl ketone hydrochloride (TLCK) (Sigma-Aldrich, Dorset, UK) for 1 h at 4°C, prior to addition to the cell cultures. The effects of this TLCK-treated \textit{P. gingivalis} preparation were compared to the ones elicited by the un-treated \textit{P. gingivalis}. To investigate the role of proteinaceous components in general, \textit{P. gingivalis} W50 culture supernatant was heat-treated at 70°C for 1 h, prior to addition to the cell cultures.

\section*{2.5 RNA extraction}

Upon termination of the experiments, the culture media were discarded and the cells were washed twice in PBS before being treated with RLT lysis buffer (QIAGEN, Crawley, UK). The collected cell lysate was homogenized with QIAshredder (QIAGEN, Crawley, UK) and total RNA was extracted by the RNeasy Mini Kit (QIAGEN, Crawley, UK), according to the manufacturer’s instructions. The extracted RNA was finally eluted in 40 µl RNase free water. The RNA concentration of the samples was determined in a NanoDrop spectrophotometer, by measuring the absorbance at 260 nm ($A_{260}$).

\section*{2.6 cDNA synthesis}

One µg of total RNA was reverse transcribed into single-stranded cDNA by using M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant), Oligo(dT)$_{15}$ Primers, and PCR Nucleotide (dNTPs) Mix, in a final volume of 15 µl, according to the manufacturer’s protocol (all from Promega, Southampton, UK). The samples and master mix were heated at 40°C for 60 min, and 70°C for 15 min, followed by cooling down at 6°C and fast-spin centrifugation. The resulting cDNA was stored at -20°C until further use.

\section*{2.7 Quantitative TaqMan® real-time PCR}

For PAR-2 gene expression analysis, quantitative TaqMan® real-time PCR was performed in an ABI Prism 7900HT Sequence Detection System and software (Applied Biosystems, Foster City, CA). 18S rRNA was used as an endogenous RNA control in the samples. The probes and the primers were synthesized by Applied Biosystems (TaqMan® Gene Expression Assay IDs PAR-2: Hs00173741-m1 and 18S rRNA: Hs99999901-s1) and the amplification reactions were performed with qPCR Master Mix (Abgene, Epsom, UK). The standard PCR conditions were 10 min at
95°C, followed 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The expression levels of PAR-2 transcripts were calculated by using the comparative Cycle threshold (Ct) method (2^(-ΔCt) formula) after normalization to 18S rRNA.

2.8 Statistical analysis
The significance of differences between the various groups was assessed by one-way analysis of variance (ANOVA) and Bonferroni post-hoc test. P values < 0.05 were considered indicative of statistical significance. The data are expressed as mean ± standard error of mean (SEM).

3. Results

3.1 Effect of P. gingivalis on PAR-2 expression in human GF
The effect of P. gingivalis on PAR-2 mRNA expression was evaluated by quantitative real-time PCR in human GF cultures. PAR-2 expression was detected in the unchallenged cells (control). After 6 h of bacterial challenge with ascending concentrations of P. gingivalis, a concentration-dependent up-regulation of PAR-2 mRNA expression was observed. This expression peaked with 12.5 µg/ml P. gingivalis, but decreased again with the highest (25 µg/ml) concentration used (Figure 1). Compared to the control, up-regulation of PAR-2 mRNA expression proved to be statistically significant by challenge with 6.25 µg/ml and 12.5 µg/ml P. gingivalis protein (4-fold and 5.6-fold, respectively). The scale of the relative PAR-2 mRNA expression was at the range of 10^{-7} to 10^{-6} in these cells.

3.2 Effect of P. gingivalis on PAR-2 expression in T-cells
The effect of P. gingivalis on PAR-2 mRNA expression was also investigated in Jurkat T-cell cultures. Compared to the unchallenged control, P. gingivalis significantly enhanced PAR-2 mRNA expression, peaking with 3.25 µg/ml, but decreased with the highest (6.25 µg/ml) P. gingivalis concentration used (Figure 2). Nevertheless, only 3.125 µg/ml P. gingivalis caused a significant up-regulation of PAR-2 mRNA expression, compared to the control (4.8-fold). The scale of relative PAR-2 expression was at the range of 10^{-4} to 10^{-3} in these cells.

3.3 Involvement of P. gingivalis cysteine proteinases
The involvement of P. gingivalis cysteine proteinases in the observed regulation of PAR-2 mRNA expression was also investigated. For this purpose, P. gingivalis culture supernatants (3.125 µg/ml) were heat-inactivated or TLCK-treated prior to challenging Jurkat T-cell cultures, in order to destroy the proteinaceous components, or inhibit the cysteine protease activity, respectively. After 6 h of challenge, both treatments resulted in abolishment of PAR-2 mRNA expression to control levels (Figure 3), which proved to be statistically significant compared to the un-treated P. gingivalis-challenged group. No statistically significant differences were observed between the control and the heat-inactivated or TLCK-treated groups (P>0.05), indicating that the responsible component for PAR-2 regulation is potentially proteinaceous and proteolytic.

4. Discussion
The present study investigated PAR-2 mRNA expression in human GF and Jurkat T-cells, with particular focus on the regulation of this expression by P. gingivalis. Under physiological conditions it was found that PAR-2 is expressed by both cell types,
although the expression level in T-cells is greater than in fibroblasts. Previous studies have reported that PAR-2 is expressed under physiological conditions in a number of cell types, including osteoblasts (Abraham et al., 2000), oral epithelial cells (Lourbakos et al., 2001a), human GF (Uehara et al., 2003), platelets (Lourbakos et al., 2001b), neutrophils (Lourbakos et al., 1998), neurons (Vergnolle et al., 2001), T-cells (Fiorucci et al., 2001), dental pulp cells (Tanchareon et al., 2005), sperm cells and oocytes (Smith et al., 2000). Nevertheless, there is no comparative evidence on quantitative differences of PAR-2 expression between cell types. The present study demonstrates that the basal level of PAR-2 expression in T-cells is greater than that in GF, indicating that PAR-2 may have distinct physiological roles, depending on the cell lineage. To this extent, PAR-2 is suggested to play multiple physiological and pathological processes, in various tissues, organs and systems (Fiorucci et al., 2001, Ossovskaya and Bunnett, 2004). In the case of inflammation, PAR-2 activation is shown to induce the production of the pro-inflammatory cytokines IL-1, IL-6 and IL-8 (Lourbakos et al., 2001, Uehara et al., 2008b, Giacaman et al., 2009).

Further on, the findings of the present study demonstrate that P. gingivalis challenge up-regulates PAR-2 mRNA expression in human GF and Jurkat T-cells. This enhancement of PAR-2 mRNA expression is likely to be an auto-regulatory effect of proteolytic activation of PAR-2 receptor on the cell membrane. Previous studies have confirmed that various P. gingivalis preparations induce PAR-2 expression or activation not only in human GF (Uehara et al., 2003), but also in oral epithelial cells (Lourbakos et al., 2001a, Uehara et al., 2002), monocytes (Uehara et al., 2008a) and neutrophils (Lourbakos et al., 1998). Therefore, the inductive effect of P. gingivalis on PAR-2 expression is not restricted to a particular cell type and appears to be universal. However, the peak of PAR-2 up-regulation in the two studied cell types occurred at a different concentration of P. gingivalis, which was 4-fold greater for GF, than for Jurkat T-cells. Interestingly, beyond this concentration, PAR-2 mRNA expression declined in both cell types, indicating that for maximal PAR-2 induction, the optimal P. gingivalis concentration is dependent on the cell type. Nevertheless, despite these differences, the magnitude of PAR-2 induction was comparable between the two cell types. Collectively, these findings indicate that T-cells and GF have quantitatively similar PAR-2 responses to P. gingivalis challenge, but the former cell type is more sensitive in this respect. This may denote that in the presence of lower P. gingivalis concentrations, the main source of PAR-2 induction in the periodontal tissues could be cells of the immune system (i.e. T-cells), whereas in presence of higher P. gingivalis concentrations that may cause death to T-cells, PAR-2 expression may be compensated by the less sensitive resident connective tissue cells (i.e. GF). This speculation is in agreement with the capacity of P. gingivalis to evade and adapt to the local immune responses, as well as to establish chronic inflammation (Lamont and Jenkinson, 1998, Dixon and Darveau, 2005, Kishimoto et al., 2006).

The present work also investigated if the cysteine proteases are the virulence factor responsible for PAR-2 induction by P. gingivalis in Jurkat T-cells. Heat-inactivation of the P. gingivalis culture supernatant resulted in total abolishment of its capacity to induce PAR-2 expression in the cells. This indicates that the responsible component is proteinaceous and minimises the possibility that the heat-stable LPS is involved. Important proteinaceous virulence factors of P. gingivalis are its gingipains. As they also possess proteolytic activity, it is tempting to postulate that they may be involved in PAR-2 induction. Indeed, treatment of P. gingivalis with TLCK, a chemical inhibitor of cysteine proteases, prior to addition to the cells resulted in total abolishment of PAR-2 induction. This is in line with a recent study in gingival epithelial cells, demonstrating that TLCK-treatment of P. gingivalis culture
supernatants diminished PAR-2 expression (Dommisch et al., 2007). Therefore, it can be deduced that gingipains could be the virulence factor responsible for PAR-2 induction by P. gingivalis. Previous studies have indicated that both Arg-X and Lys-X gingipains can activate PAR-2 (Dommisch et al., 2007, Uehara et al., 2008), but the former are more efficient in this respect (Giacaman et al., 2007, Yun et al., 2007). Nevertheless, their combined action is considered to more efficiently activate PAR-2 (Chung et al., 2004, Uehara et al., 2008).

5. Conclusions
The present study demonstrated that P. gingivalis enhances PAR-2 mRNA expression in GF and Jurkat T-cells. GF respond at higher P. gingivalis concentrations, although the magnitude of the response is similar to that of Jurkat T-cells. The virulence factors responsible for this induction are likely to be the gingipains. This induction of PAR-2 expression may play a role in the establishment of periodontal inflammation, as its activation can lead to the production of mediators of inflammation and bone destruction (Holzhausen et al., 2005b). To this extent, in vivo studies demonstrate that PAR-2-deficient mice exhibit reduced alveolar bone loss in a P. gingivalis-induced periodontitis model (Holzhausen et al., 2006), whereas topical application of a PAR-2 agonist in a rat model results in exacerbation of alveolar bone loss and gingival inflammation (Holzhausen et al., 2005a). These findings demonstrate a role of PAR-2 in periodontal disease, but also hold a good promise that PAR-2 might represent a potential target for future drug strategies.

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References


Uehara A, Imamura T, Potempa J, Travis J, Takada H. Gingipains from Porphyromonas gingivalis synergistically induce the production of proinflammatory...


Figure Legends

Figure 1. Regulation of PAR-2 mRNA expression in GF by P. gingivalis. Cells were challenged with ascending protein concentrations of P. gingivalis culture supernatants for 6 h. The mRNA expression levels of PAR-2 were measured by quantitative real-time PCR, normalized against the expression levels of 18S rRNA. The results are expressed as the $2^{-\Delta Ct}$ formula. Bars represent mean values ± SEM from four independent experiments in four GF cell lines. The asterisks indicate statistically significant difference compared to the control group.

Figure 2. Regulation of PAR-2 mRNA expression in Jurkat T-cells by P. gingivalis. Cells were challenged with ascending protein concentrations of P. gingivalis culture supernatants for 6 h. The mRNA expression levels of PAR-2 were measured by quantitative real-time PCR, normalized against the expression levels of 18S rRNA. The results are expressed as the $2^{-\Delta Ct}$ formula. Bars represent mean values ± SEM from four independent experiments with Jurkat T-cells. The asterisk indicates statistically significant difference compared to the control group.

Figure 3. Role of cysteine proteases and proteinaceous components in PAR-2 regulation by P. gingivalis. Jurkat T-cells were cultured for 6 h in the absence or presence of un-treated, heat-inactivated, or TLCK-treated P. gingivalis culture supernatant (3.125 µg/ml). The mRNA expression levels of PAR-2 were measured by quantitative real-time PCR, normalized against the expression levels of 18S rRNA. The results are expressed as the $2^{-\Delta Ct}$ formula. Bars represent mean values ± SEM from four independent experiments with Jurkat T-cells. The asterisk indicates statistically significant difference compared to the control group.
Figure 1

Relative expression

$P.\ gingivalis$ concentrations (µg/ml)

![Graph showing relative expression levels at different concentrations of $P.\ gingivalis$. The y-axis represents relative expression in arbitrary units, with values ranging from $0$ to $4 \times 10^{-6}$. The x-axis lists concentrations of $P.\ gingivalis$ at 0, 3.125, 6.25, 12.5, and 25 µg/ml. Bars indicate standard error of the mean, marked with asterisks (*) to denote statistically significant differences.](image-url)
Figure 2

![Bar graph showing relative expression of P. gingivalis concentrations (µg/ml).]
Figure 3