UNIVERSIDADE ESTADUAL PAULISTA

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MARINELLA HOLZHAUSEN

PAPEL DO RECEPTOR TIPO-2 ATIVADO POR PROTEASE (PAR-2) NA DOENÇA PERIODONTAL

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Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", do Campus de Araraquara, como requisito para a obtenção do Título de Doutor em Odontologia – Área de Periodontia.

Orientador: **Prof. Dr. Luis Carlos Spolidório**

Co-orientadora: **Profa. Dra. Nathalie Vergnolle**

Araraquara

2005

Com amor,

dedico este trabalho...

...aos meus amados pais, Joanina e Manfred.

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SUMÁRIO

PREFÁCIO

A realização desta tese baseou-se nos seguintes artigos científicos:

1. HOLZHAUSEN M, SPOLIDORIO LC, VERGNOLLE N.

Proteinase-activated receptor-2 (PAR_2) agonist causes periodontits in rats.

Journal of Dental Research

(aceito)

2. HOLZHAUSEN M, SPOLIDORIO LC, VERGNOLLE N.

Role for Protease-Activated Receptor-2 (PAR_2) in host response to

Porphyromonas gingivalis infection.

The Journal of Immunology

(submetido)

3. HOLZHAUSEN M, SPOLIDORIO LC, VERGNOLLE N.

Role of Protease-Activated Receptor-2 (PAR_2) in inflammation, and its

possible implications as a putative mediator of periodontitis

Memórias do Instituto Osvaldo Cruz

(aceito)

INTRODUÇÃO

O receptor tipo 2 ativado por protease (PAR_2) pertence a uma família recentemente descoberta de receptores acoplados à proteína G heterotrimérica. O PAR_2 pode ser encontrado em vários tecidos[1,30,35] e, sua ativação ocorre pela ação de proteases, tais como, tripsina, triptase produzida por mastócitos, proteinase tipo 3 do neutrófilo, fatores de coagulaçao VIIa/ Xa, proteinase tipo1 da membrana celular e gingipain, uma protease bacteriana produzida por *Porphyromonas gingivalis* (Pg). [26,37]

A identificação deste receptor aventa a possibilidade de um novo papel a ser desempenhado pelas proteases, não apenas como enzimas degradativas,[34] mas também como moléculas sinalizadoras capazes de influenciar as funções celulares através da ativação deste receptor.[4,15] Vários estudos sugerem que a ativação do PAR_2 desempenha um papel pró-inflamatório, levando à vasodilatação,[14,24,38] hipotensão,[14,21] edema,[33,38] migração de leucócitos,[40] e produção de citocinas inflamatórias.[6,30]

Uma possível participação do PAR_2 na doença periodontal foi sugerida por alguns estudos *in vitro*, os quais demonstraram que osteoblastos,[1] células epiteliais orais,[26,36] e fibroblastos gingivais humanos[35] expressam este receptor e que sua ativação, através da protease bacteriana gingipain[26] e de um peptídeo sintético agonista do PAR_2,[35] pode induzir a produção de mediadores inflamatórios associados à destruição dos tecidos periodontais. Levando-se em

consideração os fatos de que *Porphyromonas gingivalis* são importantes microorganismos que participam da patogênese da periodontite,[22] e de que uma de suas proteases é capaz de ativar o PAR_2, resultando em produção de citocinas pro-inflamatórias, elaborou-se a hipótese sugerindo que a ativação do PAR_2 poderia ter um papel importante nos processos inflamatórios associados com a doença periodontal.

REVISÃO DA LITERATURA

Esta revisão da literatura foi elaborada seguindo como base o seguinte artigo científico:

HOLZHAUSEN M, SPOLIDORIO LC, VERGNOLLE N. Role of Protease-Activated Receptor-2 (PAR_2) in inflammation, and its possible implications as a putative mediator of periodontitis. *Memórias do Instituto Osvaldo Cruz.*

(vide ANEXO 1)

PARs- RECEPTORES ATIVADOS POR PROTEASE

PARs: localização, ativação e função

Os receptores ativados por protease (PARs) pertencem a uma família de receptores acoplados à proteína G heterotrimérica.[29,41] Conforme ilustrado nas figuras 1A e 1B, proteases são responsáveis pela proteólise do domínio extracelular terminal- N do PAR, resultando em um novo terminal, o qual liga-se em um sítio específico no próprio receptor, desta forma, ativando-o.[30] Até o presente momento, quatro membros da família dos receptores PAR foram identificados. PAR_1, PAR_3, e PAR_4 são ativados por trombina,[12,13] e PAR_2 é ativado por tripsina, triptase produzida por mastócitos, proteinase tipo 3 do neutrófilo, fator VIIa/ fator Xa, proteinase tipo1 da membrana, ou proteases produzidas pela bactéria *Porphyromonas gingivalis*[10,26,39] (Fig. 2).

Peptídeos sintéticos seletivos, com as mesmas seqüências do terminal-N, podem ativar os receptores através de ligação direta ao corpo do receptor (fig. 1C), sem a necessidade de proteólise.[8] Com exceção do PAR3, todos os demais receptores possuem seus peptídeos agonistas seletivos. PAR_1, PAR_2, e PAR_4 podem ser ativados não-enzimaticamente por TFLLR-NH2, SLIGRL-NH2, e GYPGQV-NH2, respectivamente.[30]

Apesar de demonstrarem estruturas similares e mecanismos comuns de ativação, os PARs possuem localização tecidual e funções diferentes.[30] PAR_1 pode ser encontrado nas plaquetas humanas, endotélio, epitélio, fibroblastos, miócitos, neurônios, e astrócitos, e parece desempenhar um papel importante na

deposição de matriz vascular após injúrias. PAR3 e PAR4 são encontrados nas plaquetas, endotélio, miócitos, e astrócitos, e estão envolvidos na formação de coágulos e na embolia pulmonar.[30] PAR_2 é expresso pela maioria dos tecidos, especialmente em células epiteliais, células endoteliais, fibroblastos, osteoblastos, neutrófilos, miócitos, neurônios, e astrócitos.[1,30,35] PAR_2 parece estar envolvido na migração de leucócitos, reação alérgica das vias aéreas e inflamação de articulações, pele, e rins.[30]

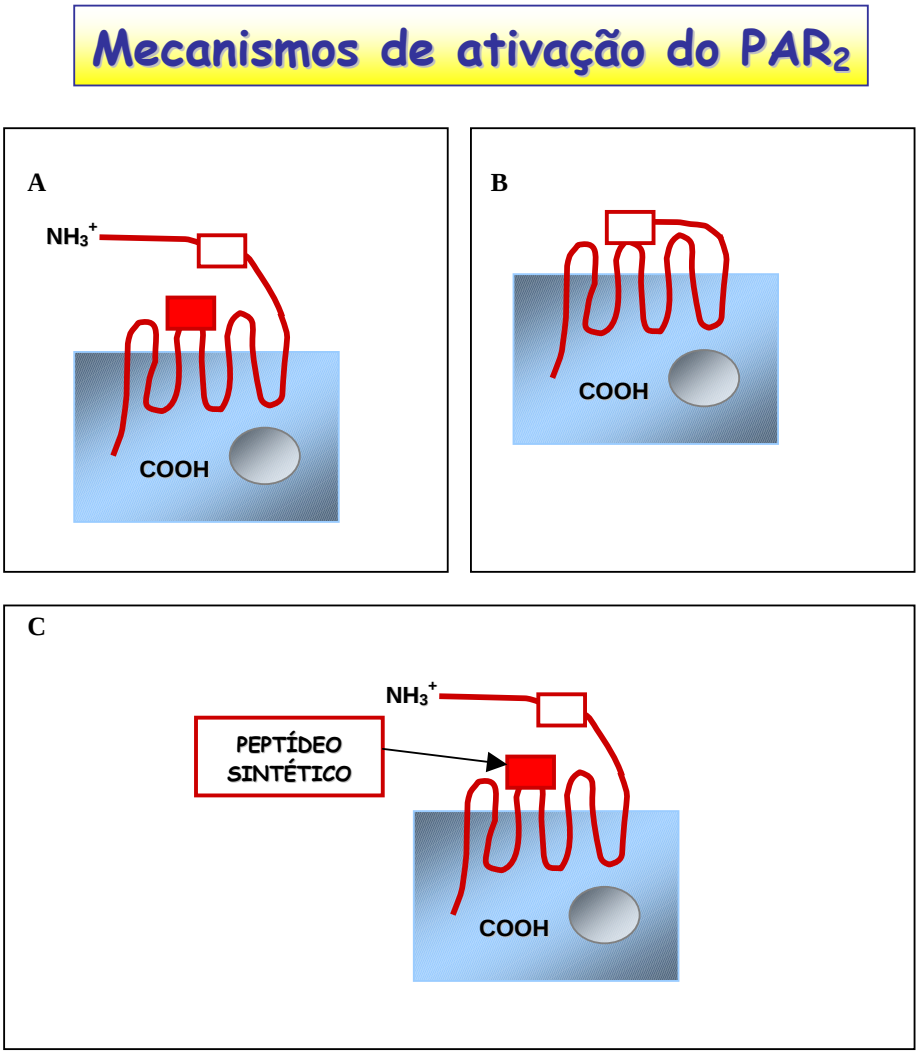

Figura 1: Mecanismos da ativação do receptor tipo-2 ativado por protease (PAR_2). Fig. 1A representa o receptor em sua forma "inativa", esperando a clivagem de seu domínio extracelular terminal-N em um sítio específico (lado esquerdo da caixa branca). Após a clivagem enzimática, o domínio extracellular terminal –N é exposto (caixa branca), e liga-se a um local específico no receptor (caixa vermelha) (1A e 1B), ativando o receptor. Peptídeos sintéticos podem também ativar o PAR_2 ligando-se diretamente ao receptor (caixa vermelha) sem a necessidade de clivagem enzimática do mesmo (1C).

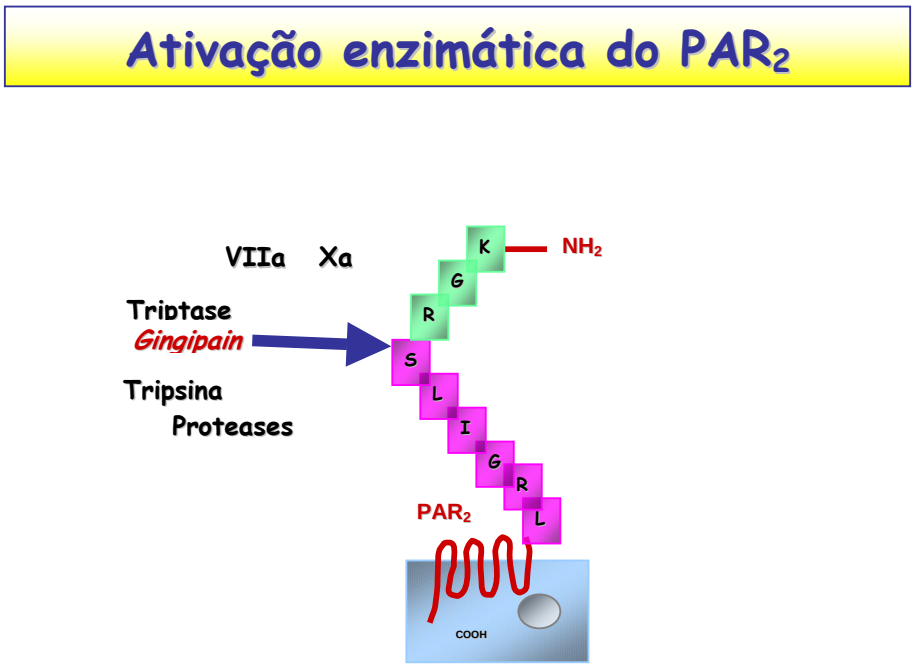

Figura 2: Mecanismo enzimático da ativação do PAR_2. Proteases endógenas tais como tripsina, triptase, fatores de coagulação VIIa e Xa, e proteases bacterianas, incluindo a protease gingipain produzida pela bactéria *Porphyromonas gingivalis*, clivam enzimaticamente em um local específico (seta azul) a seqüência do terminal-N do PAR_2.

PAR_2 & inflamação

O possível envolvimento do receptor PAR-2 na reação inflamatória foi sugerido após a verificação de que ocorre um aumento da expressão de RNAm de receptores PAR-2 em cultura de células endoteliais após estimulação com mediadores inflamatórios tais como a interleucina-1α (IL-1α) e o fator de necrose tumoral-α (TNF-α).[29] Outros estudos demonstraram que sua ativação pode levar ao relaxamento dos vasos sangüíneos,[31] hipotensão,[14] aumento da permeabilidade vascular,[23] adesão leucocitária[40] e infiltração granulocítica.[38] Além disso, ratos com deficiência genética quanto à produção de PAR-2[18] são protegidos contra a hiperreatividade na reação alérgica das vias aéreas,[32] dermatite alégica,[24] e hiperalgesia induzida por formol ou por degranulação de mastócitos.[37] Ferrel et al.,[17] sugeriram que o receptor PAR-2 pode mediar a inflamação crônica articular através da indução da liberação de citocinas pelas células endoteliais e, demonstraram que camundongos deficientes de receptores PAR-2 são protegidos contra uma forma experimental de artrite reumatóide.

A ativação do PAR_2 conduz também à liberação de metabólitos do ácido araquidônico e de citocinas tais como a interleucina IL-6 e IL-8 em células epiteliais e não-epiteliais.[26,35] PAR_2 tem perfil pro-inflamatório, podendo detectar e mediar respostas a injúrias teciduais portanto o PAR_2 desempenha papel crucial na regulação da inflamação.

Algumas células dos tecidos que compõe o trato gastrointestinal, também espressam o receptor PAR_2: enterócitos, células endoteliais da lâmina própria e da submucosa, fibroblastos, e células imunes e inflamatórias (linfócitos,

neutrófilos, mastócitos).[4,28] Recentemente, demonstrou-se que administração de agonistas do PAR_2 no colon intestinal, leva a uma reação inflamatória caracterizada por edema, infiltração granulocítica, aumento da permeabilidade intestinal e aumento de citocinas proinflamatórias (interleucina-1, TNF-alfa).[5] Estudos recentes indicam também papel importante do PAR_2 na dor.[18] O receptor foi identificado nos nervos sensoriais aferentes e foi associado a hiperalgesia térmica de longa duração[37] e a hiperalgesia no intestino de ratos.[9]

PAR_2 & inflamação periodontal

Uma possível participação do receptor PAR_2 na inflamação crônica periodontal foi sugerida por vários estudos. Primeiramente, mostrou-se que gingipain, uma protease bacteriana produzida por *Porphyromonas gingivalis* (Figura 2), um importante agente etiológico da periodontite crônica, é capaz de ativar o receptor tipo-2 ativado por protease (PAR_2).[26] Além disso, a expressão de PAR_2 foi demonstrada em osteoblastos, células do epitélio oral, neutrófilos, e em fibroblastos gengivais humanos[1,26,35] (vide Figura 3). Lourbakos et al. (2001) [26] demonstraram que em uma linhagem de células epiteliais orais, a ativação do PAR_2 pela protease bacteriana gingipain induz à secreção da citocina pro-inflamatória interleucina-6 (IL-6), a qual é um potente estimulador da diferenciação do osteoclasto e da reabsorção óssea.[2,19] Uehara et al. (2003),[35] demonstraram que um peptídeo sintético agonista do PAR_2 ativa a produção de IL-8 em cultura de fibroblastos gengivais humanos. Sabe-se que a IL-8 tem a habilidade de estimular seletivamente a atividade de matriz metaloproteinases

(MMPs) destas células, desta forma sendo responsável por parte da destruição de colágeno nas lesões periodontais.[20] Estes dois estudos sugerem um papel para a ativação do PAR_2 na indução da inflamação e na reabsorção óssea durante a periodontite.

Chung et al. (2004),[7] demonstraram que o PAR_2 está envolvido no aumento da regulação de beta-defensinas em células epiteliais gengivais humanas, estimulado pelas proteases de *Porphyromonas gingivalis*, desta forma sugerindo que a ativação do PAR_2 pode constituir-se em um mecanismos de alarme da mucosa, sinalizando a invasão de bactérias.

Desta forma a associação do receptor PAR_2 com a patogênese da periodontite é suportada por alguns conceitos:

i) PAR_2 pode ser ativado por gingipain, uma protease bacteriana produzida pelo periodontopatógeno *Porphyromonas gingivalis*;

ii) PAR_2 é expresso por células periodontais importantes como células do epitélio oral, fibroblastos, e osteoblastos, e sua ativação conduz à produção de mediadores da reabsorção óssea;

Estes achados indicam que o PAR_2 pode representar um alvo potencial para o desenvolvimento de terapias na modulação da inflamação periodontal.

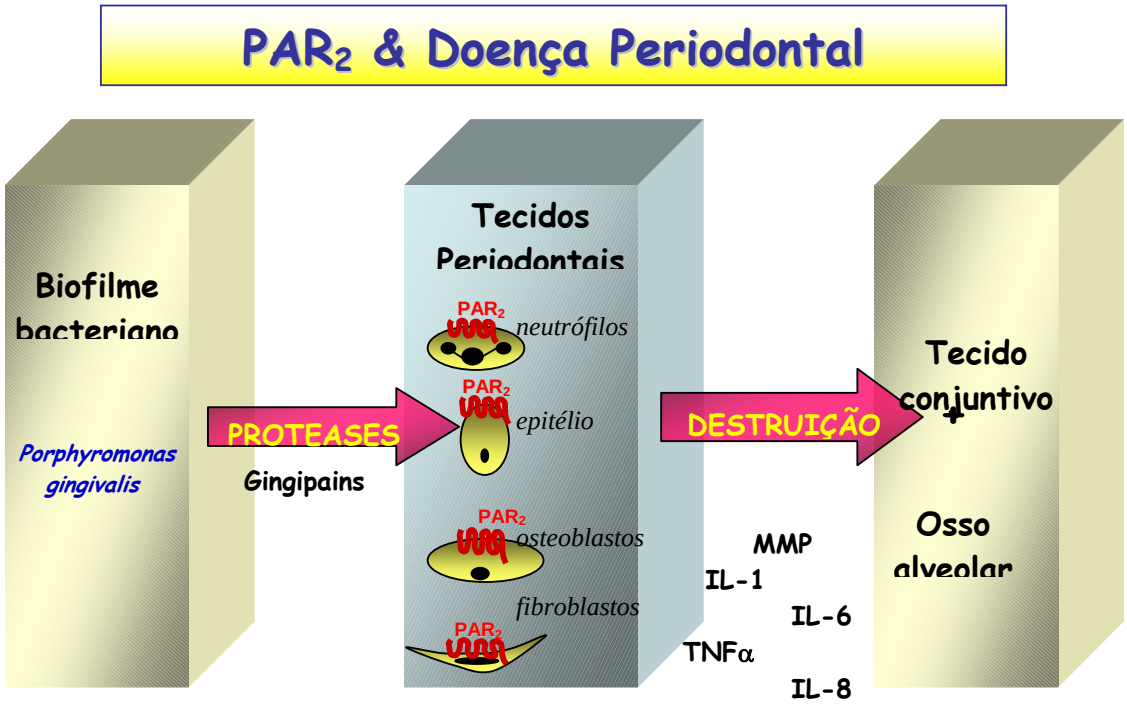

Figura 3: Possível participação do PAR_2 na doença periodontal. Gingipains produzidas pelo periodontopatógeno *Porphyromonas gingivalis*, um importante agente etiológico da periodontite do adulto, podem ativar o PAR_2 em neutrófilos, células epiteliais orais, osteoblastos, e fibroblastos gingivais, levando à produção de inúmeros mediadores pro-inflamatórios (interleucina-1: IL-1, Interleucina-6: IL-6, Fator de necrose tumoral alfa: TNFα, Interleucina-8: IL-8, matriz metaloproteinases: MMPs, que podem levar à destruição periodontal.

PROPOSIÇÕES GERAIS

Os objetivos do presente trabalho foram os seguintes:

1. Verificar os efeitos da ativação do Receptor-tipo 2 Ativado por Protease (PAR_2) sobre o periodonto de ratos, avaliando-se:

- A resposta dos tecidos periodontais após aplicação tópica gengival de um peptídeo agonista seletivo do PAR_2;
- A resposta dos tecidos periodontais após aplicação tópica gengival de um peptídeo agonista seletivo do PAR_2 em ratos submetidos à periodontite induzida por ligadura;
- Os possíveis mecanismos patológicos da inflamação gengival e perda óssea alveolar, observados após aplicação tópica do peptídeo agonista seletivo do PAR_2;

2. Verificar o papel do PAR_2 na resposta do hospedeiro à infecção por *Porphyromonas gingivalis,* avaliando-se:

- Os efeitos da infecção por *Porphyromonas gingivalis* sobre a atividade proteolítica encontrada em amostras de fluido coletadas de câmera subcutânea implantada na região dorsal de camundongos.
- Os efeitos da atividade proteolítica subsequente à infecção por *Porphyromonas gingivalis* sobre a ativação do PAR_2 em células epiteliais KNRK (KirsteN sarcoma-transformed Rat Kidney) transfectadas com o receptor PAR_2.

- Os efeitos de amostras da bactéria *Porphyromonas gingivalis* sobre a ativação do PAR_2 em células KNRK transfectadas com o receptor PAR_2.
- O possível envolvimento da ativação do PAR_2, seguida à infecção por *Porphyromonas gingivalis*, sobre a resposta inflamatória em câmeras subcutâneas implantadas em camundongos.
- O efeito da ativação do PAR_2, seguida à infecção oral por *Porphyromonas gingivalis*, sobre a perda óssea alveolar em camundongos.

CAPÍTULO 1

Proteinase-Activated Receptor-2 (PAR_2) Agonist Causes Periodontitis in Rats.

Journal of Dental Research, aceito em novembro, 2004.

Proteinase-Activated Receptor-2 (PAR_2) Agonist Causes Periodontitis in Rats

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Short title: *PAR_2 causes periodontitis*

Key Words: Proteinase-activated receptor-2; alveolar bone loss; inflammation; matrix metalloprotease; cyclooxygenase; periodontitis

Number of words in the abstract: 177

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Number of figures: 4

Number of cited references: 28

ABSTRACT

Proteinase activated receptor-2 (PAR_2) is a seven-transmembrane domain G-protein-coupled receptor that mediates cellular responses to extracellular proteinases. Since PAR_2 is expressed by oral epithelial cells, osteoblasts and gingival fibroblasts, where its activation releases interleukin-8, we hypothesized that PAR_2 activation may participate to periodontal disease *in vivo*. We have investigated the role of PAR_2 activation by SLIGRL on periodontal disease in rats. Radiographic, and enzymatic (myeloperoxydase- MPO) analysis revealed that topical application of PAR_2 agonist not only causes periodontitis but also exacerbates existing periodontitis, leading to significant alveolar bone loss and gingival granulocyte infiltration. In addition, inhibition of matrix metalloproteinase (MMP) and cyclooxygenase (COX) decreased PAR_2 agonist-induced periodontitis suggesting their involvement in the mechanism by which PAR_2 activation leads to periodontitis. More specifically, their overexpression *in vivo* in gingival tissues treated with PAR_2 agonist suggests that COX-1, COX-2, MMP-2 and MMP-9 are involved. In conclusion, PAR_2 agonist causes periodontitis in rats through a mechanism involving prostaglandin release and MMP activation. Inhibition of PAR_2 may represent in the future, a novel method that modulates the host response in periodontitis.

INTRODUCTION

The tissue breakdown resulting from periodontal disease is thought to be due, in part, to the actions of host and bacterial proteinases on periodontal tissues. It has been shown that gingivitis and periodontitis are characterized by increased levels of both host and bacterial proteolytic enzymes (Reynolds and Meikle, 1997). Among these proteinases, pathogen-derived proteolytic enzymes are responsible for tissue destruction during the course of periodontal disease. These proteinases can result in specific activation or inactivation of critical host processes, thereby affecting antimicrobial defense mechanisms or interfering with the regulation of the inflammatory reaction (Travis and Potemba, 2000). Recently, a cysteine bacterial proteinase, gingipain-R, produced by *Porphyromonas gingivalis*, a major causative agent of adult periodontitis, was reported to activate proteinase-activated receptor-2 (PAR_2) (Loubarkos *et al*., 2001). PAR_2 is a G-protein-coupled receptor activated through the proteolytic cleavage of its extracellular N-terminal domain by different proteinases including: trypsin, mast cell tryptase, neutrophil proteinase 3, tissue factor/ factor VIIa/ factor Xa, and membrane-tethered serine proteinase-1 (Vergnolle 2004). A synthetic receptor agonist peptide corresponding to the newly released N-terminal sequence that acts as a tethered ligand domain binding and activating PAR_2 (SLIGKV in human and SLIGRL on rats and mouse), is also able to activate the receptor (reviewed in Vergnolle 2004).

A role for PAR_2 during the inflammatory reaction has been suggested by several studies demonstrating that activation of PAR_2 can lead to blood vessel relaxation, hypotension, increased vascular permeability, granulocyte infiltration, and leukocyte adhesion and margination (Vergnolle 1999; Cocks and Moffat, 2000; Vergnolle 2001; Coughlin and Camerer, 2003). PAR_2 activation also leads to the release of prostanoids and cytokines including interleukin (IL)-6 and IL-8 in epithelial or non-epithelial cells (Loubarkos *et al*, 2001; Uehara et al, 2003). PAR_2 clearly can trigger inflammation *in vivo*, consistent with the notion that it may sense and mediate responses to tissue injury.

A possible participation of PAR_2 in chronic oral inflammation such as periodontitis was indirectly suggested by several studies. PAR_2 is expressed in osteoblasts, oral epithelial cells, and human gingival fibroblasts (Abraham *et al*., 2000; Loubarkos *et al*. 2001; Uehara *et al*., 2003). Lourbakos *et al*. (2001) showed that in an oral epithelial cell line, PAR_2 activation by gingipain, a trypsin-like proteinase produced by *Porphyromonas gingivalis* (Pg), induced the secretion of the pro-inflammatory cytokine interleukin-6 (IL-6), which is a potent stimulator of osteoclast differentiation and bone resorption. Uehara *et al*. (2003), demonstrated that a synthetic PAR_2 agonist peptide activates human gingival fibroblasts to produce IL-8 which has the ability to selectively stimulate MMP activity from these cells, thus in part accounting for collagen destruction within periodontitis lesions. These two studies suggest a role for PAR_2 activation in inducing inflammation and bone resorption during periodontitis. In contrast, another study by Smith *et al*. (2004) showed that PAR_2 activation by SLIGRL

inhibits osteoclast differentiation by acting on cells of the osteoblast lineage, suggesting that PAR_2 activation during periodontitis could inhibit bone resorption. These contradictory results reflect the difficulties of using *in vitro* systems to evaluate which mediators are involved in periodontal diseases. Since Pg is an important causative agent in periodontitis, and one of the proteinase released by Pg has been shown to activate PAR_2, we hypothesized that PAR_2 activation may have an important role in inflammatory processes associated with periodontitis. Therefore, we propose to study *in vivo*, the effects of PAR_2 agonist applied topically on the gingiva, either in naïve rats or during the course of periodontitis.

MATERIALS & METHODS

Animals and Surgery

Male Wistar rats (250-300 g) were obtained from Charles River Laboratories (Montreal, Quebec, Canada). The Animal Care and Ethic Committees of the University of Calgary approved all experimental protocols, which followed the guidelines of the Canadian Council on Animal Care.

To induce periodontitis, rats were anesthetized by intramuscular administration of ketamine (0.08ml/ 100g bodyweight) and xylazine (0.04ml/ 100g bodyweight), and a 3.0 silk ligature was placed around the right mandibular first molar as previously described (Holzhausen et al. 2002). Sham-operated rats were anaesthetized and treated as ligatured rats with the exception of ligature.

Treatments with PAR_2 agonist peptide (SLIGRL-NH_2) and control peptides

Using a micropipette, rats received (under light anesthesia; halothane 0.5%) a daily topical application of saline (NaCl 0.9%), control peptide LRGILS-NH_2 (1µg/day) (complete reverse sequence of PAR_2-tethered ligand, inactive on PAR_2) or PAR_2-activating peptide SLIGRL-NH_2 (1µg/day), at the mesial gingival sulcus of the right mandibullary first molar. These treatments began the same day as the surgery procedure. Rats were randomly separated into the following groups: (i) Sham + saline treatment (n=32); (ii) Sham + LRGILS-NH_2 treatment (n=32) (iii) Sham + SLIGRL-NH_2 treatment (n=32); (iv) Ligature + saline treatment (n=32); (v) Ligature + LRGILS-NH_2 treatment (n=32); (vi) Ligature + SLIGRL-NH_2 treatment (n=32). Eight animals per group were sacrificed at 3, 7, 15 and 30 days after starting daily treatments.

Bone Loss Evaluation

The distance between the cemento-enamel junction and the height of alveolar bone was determined for mesial root surfaces of lower right first molars as previously described (Holzhausen et al. 2002). Millimeters of bone loss for each radiograph were measured in a blind fashion 3 times, by the same examiner.

Myeloperoxydase (MPO) activity measurement

At sacrifice, the gingivomucosal tissues encircling the right first mandibular molars were removed and processed for MPO activity, an index of tissue granulocyte infiltration, as previously described (Cenac et al. 2002).

Effect of MMP and COX inhibition on PAR_2 agonist peptide induced alveolar bone loss and gingival granulocytic infiltration

Groups of 8 rats were treated with indomethacin (daily oral dose of 5mg/kg/day), a non-selective COX-1/COX-2 inhibitor; doxycycline (daily oral dose of 6mg/kg/day), a MMP inhibitor, or their vehicle (carboxymethylcelulose, 0.2ml/day). Then, one hour after these treatments, rats received either saline or the PAR_2-activating peptide SLIGRL-NH_2 (1µg/day), topically at the mesial gingival sulcus of the right mandibullary first molar, as described earlier. All animals were sacrificed 7 days after the beginning of treatments. MPO and alveolar bone loss were measured as described above.

Western Blot Protocol

Gingival tissues collected from animals treated with daily topical application of SLIGRL-NH_2 (1µg/day) or the control peptide LRGILS-NH_2 (1µg/day) at the mesial gingival sulcus of the right mandibullary first molars were homogenized and run on a 10% SDS polyacrylamide gel as previously described (Vergnolle et al. 1995). Membranes were incubated with anti-COX-1 (1:500), anti-COX-2 (1:500), anti-MMP2 (5µg/ml), anti-MMP9 (1:200), anti-MMP13 (1:200) or anti-TIMP1 (2µg/ml) polyclonal antibody overnight at 4^oC. The expressional changes following SLIGRL treatment were analyzed densitometrically using the molecular analyst program Quantity One from Bio Rad.

Data analysis

One-way analysis of variance (ANOVA) was used to compare means of alveolar bone loss and MPO activity among groups. In cases of significant differences

among the groups, post-hoc 2 group comparisons were assessed with Tukey-Kramer test. Unpaired t test was used to analyze the differences in the Western-blot densitometries for COX-1, COX-2, MMP-2, MMP-9, MMP-13 and TIMP-1 in the gingival tissue of SLIGRL-NH_2 or LRGILS-NH_2 treated animals. P value < 0.05 was considered statistically significant. Data are expressed as mean $\pm$ SEM.

RESULTS

Effects of PAR_2 *agonist peptide (SLIGRL-*NH_2*) on alveolar bone loss and gingival granulocytic infiltration*

Topical application of the control peptide LRGILS-NH_2 at the mesial surface of the lower right first molar did not provoke changes in alveolar bone or granulocyte infiltration measures compared to saline-treated rats at all time-points (Figures 1 A and B). In contrast, topical treatments with SLIGRL-NH_2 led to significantly ($p<0.05$) increased alveolar bone destruction when compared to saline treatments, at all time-points (3, 7, 15 and 30 days) (Fig. 1A, C and D). At 7 days, a significant increase in MPO activity, an index of tissue granulocytic infiltration, was found after SLIGRL-NH_2 treatment, when compared to the control peptide-treated or vehicle-treated groups (Fig. 1B). No differences were found in the MPO activity between saline and control peptide groups at all the observed time-points.

Effects of PAR_2 *agonist peptide (SLIGRL-*NH_2*) on alveolar bone loss and gingival granulocytic infiltration in ligature-induced periodontitis*

The satisfactory outcome of the experimental periodontitis model was confirmed, as increasing bone loss over the 30-days period after ligature was observed, with significant increases in mean bone loss from day 3 to 30 and significant increased MPO activity in all groups (Figure 2A and B). Bone loss in both saline and control-peptide groups progressed significantly between days 3 and 7 ($p<0.05$), and between days 7 and 15 ($p<0.01$) and no difference between the two groups was observed. The alveolar bone destruction at the mesial surface of the ligated first molar was exacerbated ($p<0.05$) by SLIGRL-NH_2 daily treatments when compared with treatment with the control peptide LRGILS-NH_2 treatment at experimental periods of 7, 15 and 30 days (Fig. 2A, C and D).

No difference was found in MPO activity between saline and control peptide groups at any of the experimental periods evaluated after ligature. In contrast, MPO activity in the gingival tissue of the ligated-first molars was exacerbated by SLIGRL-NH_2 treatment, compared to saline or control peptide treatment at 7 days after ligature (Figure 2B).

Effect of MMP and COX inhibition on PAR_2 *agonist peptide induced alveolar bone loss and gingival granulocytic infiltration*

Seven days of topical treatment with SLIGRL-NH_2 caused significant ($p<0.05$) increase in alveolar bone destruction and increased granulocytic infiltration when compared with treatment with vehicle alone. Indomethacin and MMP inhibitor treatments both significantly reduced PAR_2 agonist-induced alveolar bone loss

(Fig. 3A), and granulocytic infiltration (Fig. 3B), while they had no effect on the same parameters in animals that received topical treatment with saline instead of PAR_2 agonist.

Western blot analysis of COX and MMP expression in the gingival tissue of rats 7 days afterPAR_2 agonist peptide treatment

The gingival tissues from rats (n=5) that were subjected to 7 days of treatment with PAR_2 agonist showed a significant overexpression of the enzymes COX-1, COX-2, MMP-2 and MMP-9 when compared to the gingival tissues from rats (n=5) treated with the control peptide LRGILS-NH_2 (Figs 4A and 4B). Tissue expression of MMP-13 and TIMP-1 were unchanged in PAR_2-activating peptide-treated rats compared to control peptide-treated rats (Fig. 4).

DISCUSSION

Our study demonstrates that topical administration of PAR_2 agonist, SLIGRL-NH_2, but not control peptide i) induces periodontitis in rats, as observed by increased alveolar bone loss and granulocyte infiltration, and ii) exacerbates existing ligature-induced periodontitis. Moreover, we have shown that cyclooxygenase and matrix metalloprotease activities are involved as major mediators of PAR_2-induced periodontitis. These findings strongly suggest that PAR_2 plays an important role in the inflammatory process involved in the initiation and development of chronic oral inflammation such as periodontitis.

In agreement with previous *in vitro* studies which supported a destructive role for PAR_2 (Loubarkos *et al*. 2001; Uehara *et al*. 2000), our *in vivo* approach

definitively defines a pro-inflammatory and bone destruction role for PAR_2 activation in periodontal tissues.

We used myeloperoxydase activity measurement as a parameter for tissue granulocyte infiltration. Seven days after PAR_2-agonist treatment, a peak in MPO activity was observed, which decreased thereafter (see Figure 1B). As polymophonuclear neutrophils represent the main source for MPO, and because neutrophils constitute the frontline of the acute host inflammatory response, it can be concluded that PAR_2 agonist treatment lead to an acute inflammatory response. Infiltrated neutrophils can thereby release numerous mediators, including cytokines and prostaglandins. Neutrophils-mediated tissue injury and release of inflammatory mediators can promote the initiation of bone metabolism breakdown by stimulating osteoclasts (Dennison & Van Dyke, 1997). Thus, one possible explanation for the PAR_2 agonists-induced bone loss could rely, at least in part, on the induction of an acute inflammatory response.

There is compelling evidence in the literature that, pro-inflammatory cytokines not only have a direct effect on periodontal destruction but also can act indirectly by up-regulating COX-2 and MMP expression (Gemmell *et al*.1997). It can be suggested therefore that periodontal breakdown followed by exposure of gingival tissues to PAR_2 agonist, as shown by our present study, may be due to an initial stimulation of cytokine production by oral epithelial cells, resulting in matrix degradation and alveolar bone resorption through a MMP and COX-dependent pathway.

The evidence for the involvement of MMPs in PAR_2 agonist induced periodontitis was confirmed by our results showing that doxycycline administration significantly reduced PAR_2 agonist-induced alveolar bone loss and granulocyte infiltration at 7 days. Doxycycline is a non-antimicrobial tetracycline which has been shown to reduce connective tissue destruction including bone resorption in humans and/ or animal models of disease (including organ culture systems) (Crout, 1996; Golub *et al*., 1992). Inhibition of connective tissue breakdown by doxycycline can occur through different mechanisms: i) inhibition of active MMPs (collagenases, gelatinases) in the extracellular matrix, ii) prevention of the convertion of pro-MMPs into active MMPs in the extracellular matrix, and iii) downregulation of the gene expression of MMPs in epithelial, endothelial, and bone cells (Lee *et al.* 2004). In the present study, a significant overexpression of MMP-2 and MMP-9 was found in gingival tissues of rats 7 days after topical treatment with the PAR_2 agonist. Considering the fact that MMP-2 and MMP-9 are capable to degrade the organic component of bone (Mansell *et al*., 1997), it is thereby reasonable to think that doxycycline reduces PAR_2-induced periodontitis by inhibiting MMP-2 and MMP-9 overexpression.

The results from our experiments also evidenced the involvement of COXs in PAR_2 agonist-induced periodontitis as non-selective inhibition of COX enzymes by the non-steroidal anti-inflammatory agent indomethacin lead to a significant decrease in the alveolar bone loss and granulocyte infiltration. In addition, SLIGRL treatment induced an overexpression of COXs in the gingival tissues. It is known that the type-2 isoform of the enzyme cyclooxygenase (COX), COX-2,

along with the type-1 isoform (COX-1), regulates a variety of physiological and pathological processes through the synthesis of prostaglandins, prostacyclin and thromboxanes from arachidonic acid (Seibert, 1994). At sites of injury and inflammation, macrophages, and fibroblasts express COX-2 subsequently up-regulating the production of prostaglandin E_2. As PGE_2 is an important pro-inflammatory mediator in gingivitis and alveolar bone resorption (Lohinai *et al*., 2001), its possible overproduction by PAR_2 agonist-induced COX-2 overexpression in gingival tissues may constitute another possible pathway by which SLIGRL induces periodontal disease. It is generally admitted that while COX-2 expression is up-regulated upon inflammatory stimulation, the expression of COX-1 is constitutive. In our study, we observed that not only COX-2, but also COX-1 expression was up-regulated in gingival tissues (see Figure 4). A possible explanation is that the increased COX-1 expression is due to the massive periodontal infiltration of granulocytes, which constitutively express COX-1 (McAdam *et al.* 2000). However, overexpression of COX-1 has been demonstrated in different cell types (mast cells, monocytes in response to lipopolysaccharide) (Smith *et al.* 1996, Smith & Dewitt, 1996, Parente & Perretti, 2003), therefore, we cannot rule out the possibility that COX-1 is overexpressed by a specific cell type in response to PAR_2 agonist.

Human periodontal disease is always associated with an infectious component. *Porphyromonas gingivalis* has been recognized as the major periodontopathogen responsible for human chronic periodontitis. Although the use of *P. gingivalis*-infection model in rats has advantages, specifically because it is very close to the

human disease and because it involves numerous proteases released by pathogens, we choose to use a mechanically-based model of periodontitis. The major reason to choose this model was from the fact that the numerous proteases released by *P. gingivalis* could have caused degradation of the PAR_2-activating peptide, thereby masking the potential enhancing effects of PAR_2 activation on periodontitis. The model we used, which consist in the placement of a ligature around the cervix of the first mandibullary molar, is a very well established model of periodontitis,. The ligature acts not only by causing a mechanical trauma on the dentogingival area, but also by promoting plaque accumulation, thus increasing the number of bacteria (Lohinai *et al*., 1998). This model, takes into account an important part of the representation of the human disease: the intense host-plaque interactions.

The gingival sulcus can be an environment rich in proteases derived from periodontopathogens. These highly proteolytic enzymes have been implicated as important factors in eliciting host responses that result in the destruction of periodontal tissues (Ekuni *et al*.,2003). The exact mechanism by which bacterial proteases mediate periodontitis is far from being understood in details. Based on the results obtained from the present study, one possible explanation could be that bacterial proteases can mediate periodontal breakdown through the activation of the PAR_2 receptor in host cells. This hypothesis is further supported by the fact that gingipain, a protease released by *P. ginvivalis*, has been shown to activate PAR_2 *in vitro* (Loubarkos *et al*. 2001). However, we cannot rule out the possibility that PAR_2 in host gingival tissues might be activated by host

proteases such as trypsin, which can be released by damaged endothelial cells, or tryptase, which is released upon mast cell degranulation.

In conclusion, we have shown that PAR_2 activation in periodontal tissues constitutes a destructive signal, causing all the signs of periodontitis. Since PAR_2 can be activated by periodontal pathogen proteases (Loubarkos *et al*. 2001), these results strongly suggest a role for PAR_2 activation in the development of periodontal diseases. Inhibition of PAR_2 activation or even proteolytic activity may thus represent a novel therapeutic alternative, particularly for the treatment of the most aggressive forms of periodontitis. PAR_2 inhibition could constitute an alternative approach to modulate host response to periodontal pathogens.

ACKNOWLEDGMENTS

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FIGURES

Figure 1

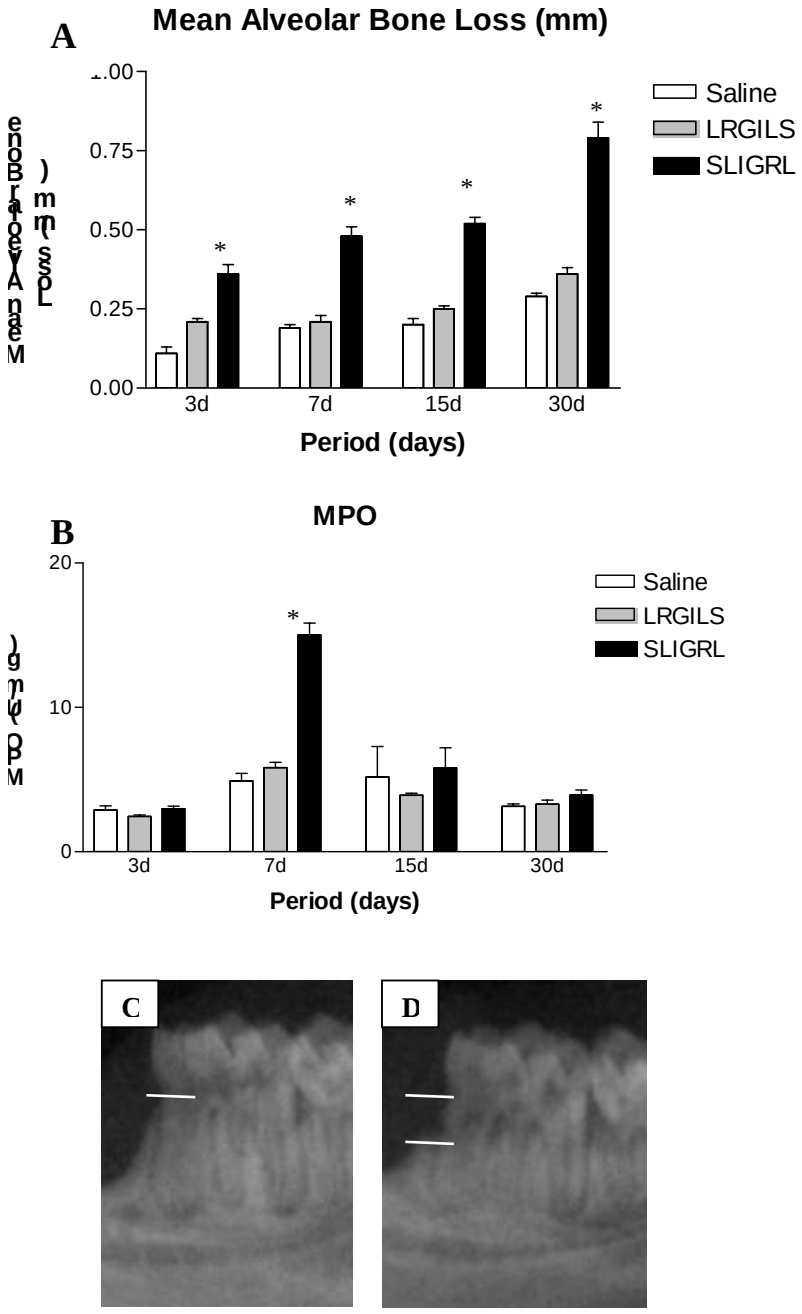


Figure 2

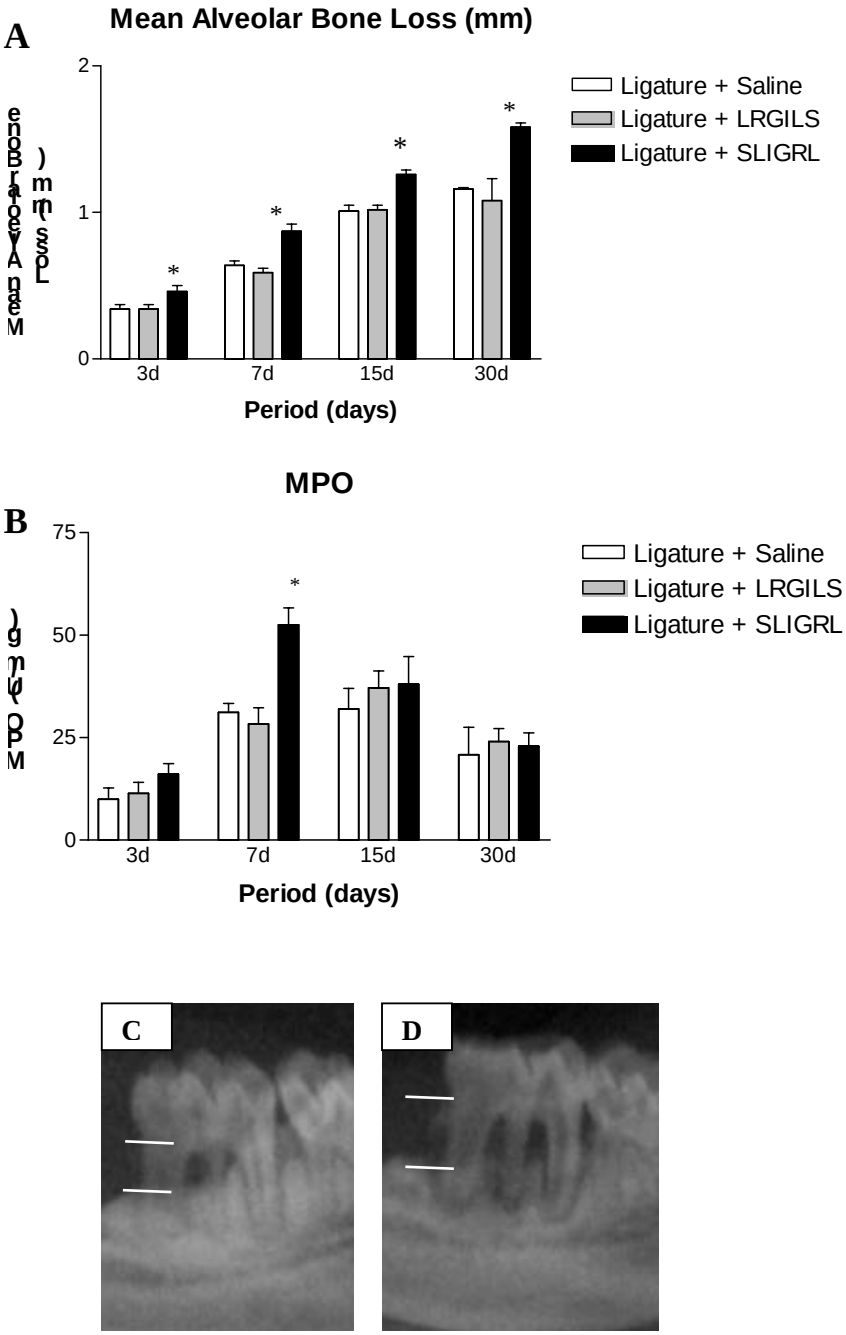

A
Mean Alveolar Bone Loss (mm)
Alveolar Bone Loss (mm)
2
1
0
3d
7d
15d
30d
Period (days)
Ligature + Saline
Ligature + LRGILS
Ligature + SLIGRL
B
MPO
MPO (mU/mg)
75
50
25
0
3d
7d
15d
30d
Period (days)
Ligature + Saline
Ligature + LRGILS
Ligature + SLIGRL
C
D

Figure 3

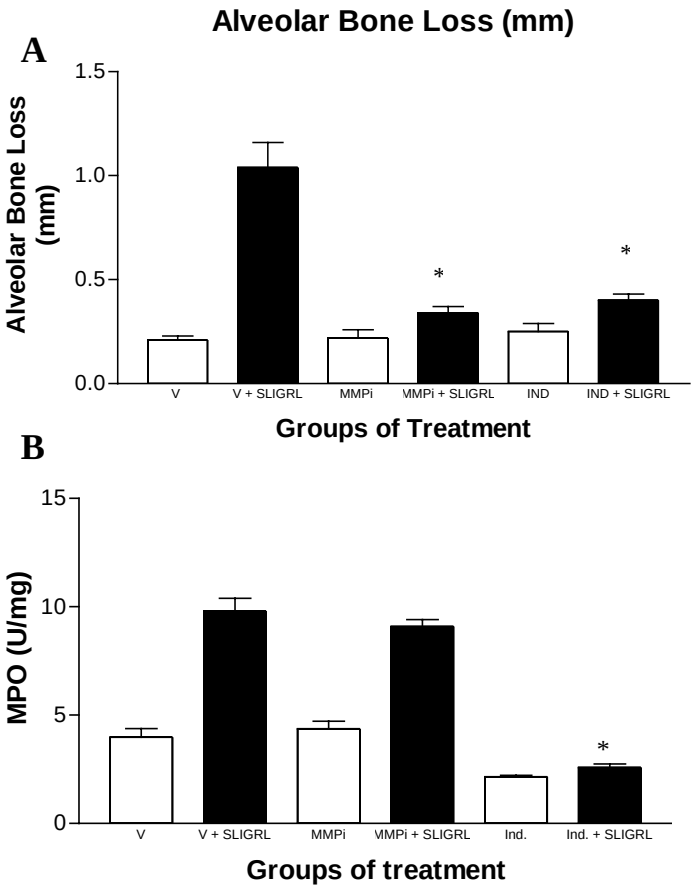
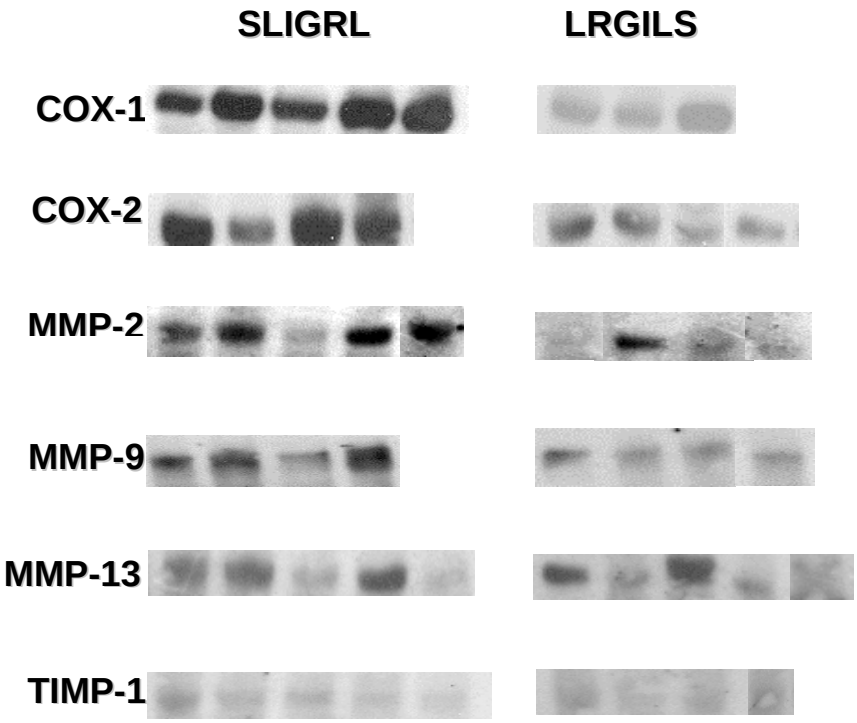

Alveolar Bone Loss (mm)
A
Alveolar Bone Loss (mm)
1.5
1.0
0.5
0.0
*
*
V
V + SLIGRL
MMPi
MMPi + SLIGRL
IND
IND + SLIGRL
Groups of Treatment
B
MPO (U/mg)
15
10
5
0
*
V
V + SLIGRL
MMPi
MMPi + SLIGRL
Ind.
Ind. + SLIGRL
Groups of treatment

Figure 4

A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LRGILS
COX-1
COX-2
MMP-2
MMP-9
MMP-13
TIMP-1

B

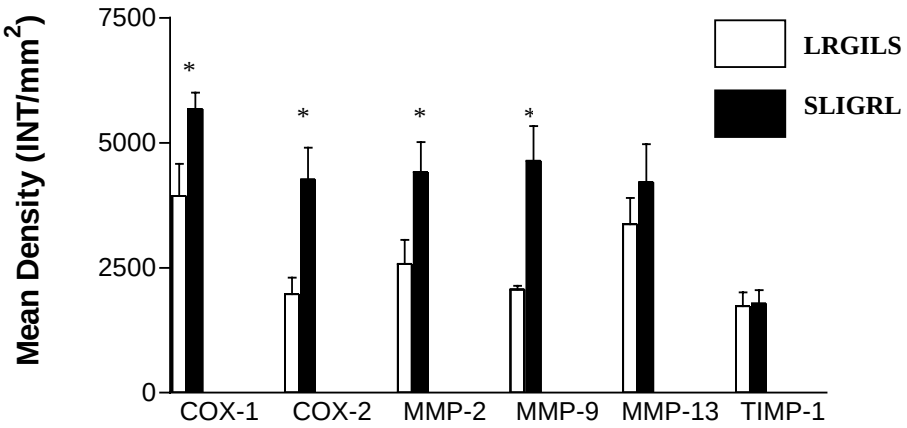
Mean Density (INT/mm2)
7500
5000
2500
0
LRGILS
SLIGRL
COX-1
COX-2
MMP-2
MMP-9
MMP-13
TIMP-1

FIGURE LEGENDS

Figure 1- *Effects of topical gingival application of* PAR_2 *agonist peptide (SLIGRL-*NH_2*) control peptide (LRGILS-*NH_2*), or saline, on radiographic alveolar bone loss (mm) (**A**) and granulocyte infiltration (**B**), at 3, 7, 15 and 30 days after beginning the treatment.* *significant difference (P<0.05) compared with group Saline at the same time period (ANOVA). N=8 animals/group/period; data expressed as Mean ± SEM. *Representative digital radiograph of the mandibular area 30 days after daily treatment with control peptide LRGILS-*NH_2 *(**C**) or* PAR_2*-activating peptide SLIGRL-*NH_2 *(**D**) showing preservation (**C**) or resorption (**D**) of bone crest at the mesial surface of the first molar and in the furcation area.* In C and D, the upper white lines represent the anatomical point cemento-enamel junction, and the lines positioned in the botton, represent the alveolar bone crest. The distance between these two reference points represents the alveolar bone loss at the mesial surface of the first mandibullary molar.

Figure 2- *Ligature-induced periodontitis, effects of topical gingival application of* PAR_2 *agonist peptide (SLIGRL-*NH_2*) control peptide (LRGILS-*NH_2*), or saline, on radiographic alveolar bone loss (mm) (**A**) and granulocyte infiltration (**B**), at 3, 7, 15 and 30 days after ligature and the beginning of the treatment.* *significant difference (P<0.05) compared with group Ligature + Saline group at the same time period (ANOVA). N=8 animals/group/period; data expressed as Mean ± SEM. *Representative digital radiograph of the mandibular area 30 days after ligature placement and the beginning of daily treatment with control peptide LRGILS-*NH_2 *(**C**) or* PAR_2*-activating peptide SLIGRL-*NH_2 *(**D**)* showing resorption

of bone crest at the mesial surface of the first molar and in the furcation area due to the presence of ligature (**C**) and exacerbated resorption when ligature was combined with PAR_2 agonist peptide treatment (**D**). In C and D, the upper white lines represent the anatomical point cemento-enamel junction, and the lines positioned in the botton, represent the alveolar bone crest. The distance between these two reference points represents the alveolar bone loss at the mesial surface of the first mandibullary molar.

Figure 3- *Effects of MMP inhibitor (MMPi), Indomethacin (Indo)or their vehicle (V) treatments on PAR_2 agonist peptide(SLIGRL)-induced alveolar bone loss (**A**) and granulocyte infiltration (**B**), at 7 days after the beginning of PAR_2 agonist treatment.* *significant difference (P<0.05) compared with group V + SLIGRL at the same time period (ANOVA).

Figure 4- *Expression of MMPs and COX enzymes in gingival tissues of rats 7 days after the beginning of the PAR_2 agonist (SLIGRL) or control peptide (LRGILS) daily topical treatment analyzed by Western-blot. Representative Western-blots for MMPs and COX enzymes (**A**), each band corresponds to the tissues from one rat. Mean density of detected bands (B),* N=5 animals/group; data expressed as Mean ± SEM. *****Significant differences (p<0.05) when compared with group LRGILS by Unpaired t test.

CAPÍTULO 2

Role for Protease-Activated Receptor-2 (PAR_2) activation in host response to *Porphyromonas gingivalis* infection

The Journal of Immunology, submetido (Dezembro, 2004).

Role for Protease-Activated Receptor-2 (PAR_2) activation in host response to *Porphyromonas gingivalis* infection

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Short title:

Key Words: Protease-Activated Receptor-2; alveolar bone loss; inflammation; host response, *Porphyromonas gingivalis*;

ABSTRACT

Protease-Activated Receptor-2 (PAR_2) is a G-protein-coupled receptor that mediates *in vitro* cellular responses to extracellular tryptic proteases, such as gingipain, a protease produced by the periodontal pathogen *Porphyromonas gingivalis* (Pg). We have investigated the specific contribution of PAR_2 to host defense during Pg infection. Injection of Pg into subcutaneous chambers in mice provoked in fluids collected from the chambers an increased proteolytic activity, which was inhibited by the addition of a soybean trypsin inhibitor (SBTI). Fluids collected from Pg-treated mice provoked a calcium signal in KNRK cells transfected with PAR_2 but not in non-transfected cells or in cells that were desensitized by pre-exposure to trypsin. Moreover, Pg inoculation into subcutaneous chambers of wild-type mice induced an inflammatory response of the host characterized by a significant increase in inflammatory cell infiltration, which was inhibited by SBTI, and significantly reduced in PAR_2-deficient ($PAR_2^{-/-}$) mice. Increased levels of prostaglandin E_2, interferon-gamma, interleukin-6 and interleukin-1β were found in chambers of Pg-treated mice compared to control saline-treated mice. Except for interleukin-6, all these inflammatory mediators were either not increased or significantly inhibited in $PAR_2^{-/-}$ mice infected with Pg. Mice orally challenged with Pg developed periodontitis characterized by important alveolar bone loss. This increased alveolar bone loss was significantly reduced in $PAR_2^{-/-}$ mice at 42 and 60 days after Pg infection. These results clearly show that PAR_2 is activated upon Pg infection, where it plays an important role in the host inflammatory response. Inhibition of PAR_2 may represent in the

future, a novel therapeutic approach to modulate the host response in periodontitis.

INTRODUCTION

The protease-activated receptors (PARs) belong to a recently described family of G-protein-coupled, seven-transmembrane-domain receptors (1, 2). Activation of PARs occurs through proteolytic cleavage of the extracellular domain, resulting in generation of a new N-terminal “tethered ligand” (3). To date, four PARs have been identified: PAR_1, PAR_2, PAR_3, and PAR_4 (4, 5). For PAR_1, PAR_2, and PAR_4, synthetic peptide agonists corresponding to the newly created N-terminus, are able to activate the receptor in the absence of receptor cleavage (6, 7).

Although the receptors have similar structures, they have different roles and tissue distribution and they can be activated by different proteases. PAR_1, PAR_3, and PAR_4 are activated by thrombin and a role in platelet activation has been described. Trypsin, mast cell tryptase, neutrophil protease 3, tissue factor/ factor VIIa/ factor Xa, membrane-tethered serine protease-1, and proteases from *Porphyromonas gingivalis* have been identified as activators of PAR_2 (8, 9, 10), which is widely distributed throughout the body, especially in the gastrointestinal tract. Several studies have shown that PAR_2 participates in the inflammation process *in vivo*, consistent with the notion that it may sense and mediate responses to tissue injury (8, 9, 10, 11, 12, 13).

Recently, a number of studies have linked PAR_2 to periodontitis, a chronic oral inflammation. Loubarkos *et al*., 2001 (8), reported that a cysteine bacterial protease, gingipain-R, produced by *Porphyromonas gingivalis*, a major causative agent of adult periodontitis, was able to activate protease-activated receptor-2 (PAR_2). This activation induced the secretion of the pro-inflammatory cytokine interleukin-6 (IL-6), which is a potent stimulator of osteoclast differentiation and bone resorption. The production of potent pro-inflammatory mediators was also shown by Uehara *et al*., 2003 (13), who demonstrated that a synthetic PAR_2 agonist peptide activates human gingival fibroblasts to produce IL-8 and to selectively stimulate matrix metalloproteinase (MMP) activity from these cells, thus in part accounting for collagen destruction within periodontitis lesions. In addition, PAR_2 is expressed in osteoblasts, oral epithelial cells, and human gingival fibroblasts (8, 13, 14). Moreover, in a previous study (15), we have evaluated the role of PAR_2 activation by a selective agonist (SLIGRL) on periodontal disease in rats, showing that PAR_2 agonist causes periodontitis (inflammation and alveolar bone loss) through a mechanism involving prostaglandin release and MMP activation. Taken together, these studies strongly suggest a role for PAR_2 activation in inducing inflammation and bone resorption during periodontitis. However, mainly because of the lack of available PAR_2 antagonists, no study has investigated the role of PAR_2 to the host defense against pathogens. Therefore, we used a genetic approach with PAR_2-deficient mice, to study the contribution of PAR_2 activation in an infectious model.

In the present study we evaluated the specific role of PAR_2 in the host defense against *Porphyromonas gingivalis* by using a subcutaneous chamber model of local infection and an experimental periodontitis model in wild-type (WT) and PAR_2-deficient (PAR_2 -/-) mice.

MATERIALS & METHODS

Animals

PAR_2 deficient (PAR_2-/-) and wild-type littermate (PAR_2+/+) mice, with approximately 8 weeks, were obtained from the Johnson & Johnson Pharmaceutical Research Institute (Spring House, PA). All of the animals were housed in a temperature-controlled room; food and water were provided *ad libitum*. The Animal Care and Ethic Committees of the University of Calgary approved all experimental protocols, which followed the guidelines of the Canadian Council on Animal Care.

Bacteria

Porphyromonas gingivalis strain ATCC 33277 (Rockville, MD) was grown on anaerobic blood agar plates in an anaerobic chamber with 85% N_2, 5% H_2, and 10% CO_2. After incubation at 37^0C for 7 days, the bacterial cells were collected and suspended in Schaedler broth (Difco Laboratories, Detroit, Mich.) to a final optical density of 1.2 (10^9 CFU/ml) at 660 nm.

Subcutaneous chamber

Coil-shaped chambers were prepared from 0.5-mm stainless-steel wire and surgically implanted in the subcutaneous tissue of the dorsolumbar region of

each mouse, as previously described (16). Ten days after implantation, chambers were inoculated with 0.1 ml of *P.gingivalis* suspended in Schaedler broth (10^9 CFU/ml). Control mice were inoculated with vehicle only. Mice were sacrificed by cervical dislocation at 1 day after bacterial inoculation.

Another group of mice was inoculated with 0.1 ml of a solution containing *P.gingivalis* (10^9 CFU/ml) and 0.3 mg/ml of SBTI (soybean trypsin inhitor; Sigma, St. Louis, MO, USA), and sacrificed 1 day after.

Subcutaneous chamber fluid analysis-Host response

A sample of chamber fluid (100µl) was aseptically collected from each animal at 1 day post-*P. gingivalis* challenge to assess the inflammatory response. A 10µl aliquot of each chamber fluid was 10-fold diluted in Turk's staining to determine the total number of inflammatory cells by using a Neubauer counting chamber under a light microscope.

Proteolytic activity

After centrifugation of the samples, the supernatant was collected and assayed for its proteolytic activity. Briefly, hydrolysis of substrates containing the AMC (7-amino-4-methyl coumarin) fluorophore was carried out in microtitre plate format using a Fluoroskan Ascent fluorimeter (Labsystems OY). Assays were performed at 25 °C, with a 355 nm excitation wavelength filter and a 460 nm emission wavelength filter. Fluorescence from wells on the microtitre plate was measured at 20 s intervals over 30 min. The system was calibrated with known amounts of the AMC hydrolysis product in a standard reaction mixture. All assays were

carried out in triplicate; the range of values observed was always less 10% of the mean.

Measurement of Prostaglandin-E_2, Interferon-gamma, Interleukin-1, Interleukin-6, and interleukin-10

Prostaglandin-E_2, Interferon-gamma, Interleukin-1, Interleukin-6, and interleukin-10 levels in the supernatants of chamber fluid samples collected from mice that received inoculation of *P.gingivalis* or vehicle were determined by using commercially available ELISA kits according to manufacturer's instructions (R&D Systems, Minneapolis, Minn.). The concentration of the inflammatory mediators was determined using the Softmax data analysis program (Molecular Devices, Menlo Park, CA).

Cell culture

PAR_2-expressing Kirsten sarcoma-transformed rat kidney epithelial cells (KNRK: American Tissue Type Culture Collection, Manassas, VA), and non-transfected KNRK cells were propagated in Geneticin (0.6 mg/ml)-containing medium (Dulbecco's modified Eagle's medium, 10% FBS, 1%Penstrep). Cells at 90% confluence in 80-cm^2 flasks (Life Technologies, Inc.) were rinsed with PBS, lifted with nonenzymatic cell dissociation fluid, and pelleted before resuspension in 1ml of Hank's balanced salt solution (HBSS, ph 7.4), 2.5µl of sulfinpyrazone (100mM), 1µl of a 20% Pluronic F-127 solution, and 10µl of 2.5 mg/ml Fluo-3 acetoxymethylester (Molecular Probes, Inc., Eugene, OR). The final solution was incubated at room temperature while shaking gently for 25 min. Cells were then washed three times and resuspended in calcium assay buffer (150mM NaCl,

3mM KCl, 1.5mM $CaCl_2$, 10mM glucose, 20mM HEPES, 0.25 sulfinpyrazone, pH 7.4).

Calcium signaling assay

Calcium signaling was performed as described previously (17). Briefly, fluorescence measurements were performed on a PerkinElmer fluorescence spectrometer 650-10S (Norwalk, CT, USA), with an excitation wavelength of 340nm and emission recorded at 380nm. Cell suspensions (2ml of 3 X 10^5 cells/ml) in 4ml-cuvettes were stirred with a magnetic flea bar and maintained at 24^{o}C.

The signal produced by PAR_2-expressing KNRK or non-transfected KNRK cells was measured after the addition of 40µl of *P.gingivalis* (suspended in Schaedler broth, 10^9 CFU/ml), or 2nM trypsin (Sigma, St. Louis, MO, USA), or 2µM of SLIGRL-NH_2. The peptide was synthesized by the Peptide Synthesis Facility (University of Calgary, Calgary, AB, Canada). The same experiments were also performed with PAR_2-expressing KNRK cells, and the addition of the supernatant of chamber fluid samples, or *P. gingivalis* samples previously incubated with 10 nM of SBTI for 10 minutes.

All experiments were repeated three to five times, with at least 8 samples.

Oral Infection

A total of 0.2 ml of *P. gingivalis* (10^9 CFU) was given to each mouse via a feeding needle every other day for a total of 4 days, in part by gavage, and by local application in the oral cavity. Mice were then allowed free access to standard mouse chow and water.

The mice were sacrificed with cervical dislocation 42 and 60 days after the last bacterial administration. Ten sham-infected and ten infected mice were used at each time point for each animal group (PAR_2-/- or WT mice). Mandibles were removed, hemisected, exposed to NaOH (2N), and then mechanically defleshed.

Alveolar bone loss

Horizontal bone loss around the mandibullary molars was assessed by measuring the distance between the cementoenamel junction (CEJ) and alveolar bone crest (ABC) with the aid of a dissecting microscope (x40). Measurements of bone level were done at seven sites on the lingual side of the left and right mandibullary molars, and a total of 14 measurements per mouse were done three times in a random and blinded protocol by one evaluator.

Data analysis

One-way analysis of variance (ANOVA) was used to compare means among groups. In case of significant differences among the groups, post-hoc 2 group comparisons were assessed with Tukey-Kramer test. P value < 0.05 was considered statistically significant. Data are expressed as mean $\pm$ SE.

RESULTS

P. gingivalis infection increased proteolytic activity

Because earlier studies showed that Pg protease, gingipain, is able to activate PAR_2 *in vitro*, we hypothesized that PAR_2 can be activated upon Pg infection. To this end, we first evaluated whether or not Pg infection provokes an increase in the proteolytic activity. Results in Figure 1 show that WT mice inoculated with *P.*

gingivalis showed a significant ($p<0.05$) increase in the fluid proteolytic activity when compared to saline treatment at 1 day. In addition, SBTI treatment significantly decreased the proteolytic activity due to *P. gingivalis* inoculation (Fig.1). No differences were found regarding the proteolytic activity of samples from saline and SBTI-*P.gingivalis* treated WT mice. Thus, an increased enzymatic activity is found in fluid samples from Pg infected mice.

PAR_2 is activated by P. gingivalis infection-induced proteolytic activity

To determine if this proteolytic activity could cleave PAR_2, we examined the effects of Pg infected chamber fluid samples on intracellular calcium ion concentration $[Ca^{2+}]_i$ in cultured PAR_2-KNRK cells. Results in Figure 2A show that chamber fluid samples (40µl) collected from WT mice after subcutaneous challenge with *P. gingivalis* decreased the subsequent calcium response by trypsin in KNRK cells expressing rat PAR_2 (46.33% of reduction), thus providing evidence that both are able to activate the same receptor. No responses in PAR_2-expressing KNRK were verified with the addition of fluid samples collected from control animals. In addition, non-transfected KNRK cells did not respond to the addition of fluid samples from *P. gingivalis* infected animals (Fig.2A).

Moreover, fluid samples collected from WT mice treated with both *P. gingivalis* and soybean trypsin inhibitor (SBTI) lead to a minimal response, and decreased only 30.62% the subsequent calcium response by SLIGRL-NH_2 (Fig.2B), indicating a requirement for enzymatic activity (Figure 2B).

We also examined the effects of culture supernatants of Pg on Ca^{2+} mobilization in PAR_2-KNRK cells in order to evaluate whether the proteolytic activity produced

after Pg infection was deriving from the bacterium itself or from the host proteases (Figure 3).

We first demonstrated that in the wild KNRK cell line, no responses were verified after addition of *P. gingivalis* culture supernantant samples aliquots (Fig.3A) or trypsin (Fig.3B). Then, we showed that *P. gingivalis* culture supernatant samples (40µl) lead to Ca^{2+} mobilization in PAR_2-KNRK cells, decreasing the subsequent responses to SLIGRL-NH_2 or trypsin (see Figs. 3C, 3D, 3F, 3G).

In addition, *P. gingivalis* samples pretreated with SBTI for 10 min failed to increase $[Ca^{2+}]_i$ on KNRK cells expressing PAR_2, and did not interfere with the calcium response lead by the subsequent addition of SLIGRL-NH_2 (Fig. 3E). Moreover, it was verified that after desensitization of the receptor by 2 subsequent treatments with trypsin.for 5 min, the addition of *P. gingivalis* culture supernatant samples did not lead to any calcium response in KNRK cells expressing rat PAR_2 (Fig.3H). Thus, taken together, these results provide evidence that PAR_2 activation is mediated by the enzymatic activity of *P. gingivalis*.

PAR_2 activation plays a pivotal role in the host response to P. gingivalis infection

We evaluated whether PAR_2 activation mediates the host response to *P. gingivalis* infection by comparing the inflammatory effects of subcutaneous chamber infection with Pg in WT and PAR_2-/- mice. Figure 4A shows that subcutaneous challenge of WT mice with *P. gingivalis* led to significantly ($p<0.05$) increased inflammatory cell count when compared to saline treatment. At 1 day

after *P. gingivalis* inoculation, significant increased inflammatory cell counts were also observed in WT mice compared to PAR_2-/- mice. No differences were found in the inflammatory cell count between saline and *P. gingivalis* PAR_2-/- mice (Fig 4A).

Moreover, subcutaneous challenge of WT mice with *P. gingivalis* led to significantly ($p<0.05$) increased PGE_2 levels when compared to saline treatment (Fig.4B). No significant differences were observed between WT mice compared to PAR_2-/- mice. Interferon-gamma (IFN-γ) levels were significantly increased in *P.gingivalis* - treated WT mice when compared to saline- treated WT mice, and compared to *P.gingivalis* - treated PAR_2-/- mice (Fig.4C).

Furthermore, significant IL-1β levels were found in WT mice treated with *P. gingivalis* when compared to saline- treated WT mice, and compared to *P. gingivalis* - treated PAR_2-/- mice (Fig.4D). We also showed that *P. gingivalis* treatment led to significant ($p<0.05$) increases in the IL-6 levels when compared to saline treatment in WT and PAR_2-/- mice (Fig.4E).

Regarding the IL-10 levels present in the fluid collected from the subcutaneous chambers, no significant differences were found between the saline and *P. gingivalis* treated WT and PAR_2-/- mice (Fig.4F). Interestingly, it was verified a trend (not significant) for decreased levels of IL-10 in the *P. gingivalis* treated animals when compared to saline groups.

Thus, PAR_2 activation mediates the host response to *P. gingivalis* infection in the anaerobic chamber model in mice.

PAR_2 activation plays a pivotal role in P. gingivalis-induced periodontitis

We conducted the next experiments in order to evaluate whether PAR_2 activation plays a role in *P. gingivalis* induced periodontitis in mice. The results showed that the satisfactory outcome of the experimental periodontitis model was confirmed, as increasing bone loss over the 42 and 60-days period after *P. gingivalis* administration was observed, with significant increases in mean bone loss compared to saline-treated animals in all groups (WT and PAR_2-/-). However, WT mice showed significant higher mean alveolar bone loss compared to PAR_2-/- mice at 42 and 60 days after oral infection. See data shown in figures 5A and 5B, respectively, 42 and 60 days.

DISCUSSION

The proteolytic activity of some endogenous or bacterial proteases is able to cleave the N-terminal peptide of PAR_2, providing a newly exposed N-terminal end which functions as a “tethered ligand”, activating the receptor and resulting in intracellular signaling by G_q proteins. Pancreatic and extrapancreatic (endothelial and epithelial) trypsins, coagulation factors (FVIIa-FXa), mast cell tryptase, leukocyte proteases (neutrophil proteinase 3), and membrane-tethered serine protease-1, are some of the endopeptidases that have been found to cleave and activate PAR_2 (8, 9, 10). In addition to endogenous host enzymes, the bacterial arginine-specific gingipain-R (RgpB) derived from *Porphyromonas gingivalis* (Pg), the major mediator of periodontitis, has also been identified as an important activator of PAR_2. Some *in vitro* studies have reported that RgpB can activate

PAR_2 in human neutrophils (18), human oral epithelial cells (8) and in primary-rat calvarial osteoblast-like cells (14), through the induction of a dose-dependent increase in $[Ca^{2+}]_i$.

Our present study showed that Pg culture supernatant samples lead to Ca^{2+} mobilization on KNRK cells expressing PAR_2, decreasing the subsequent responses to SLIGRL-NH_2 or trypsin. Similarly to Lourbakos et al., 2001 (8) in our present study we used transfected cells, therefore allowing a more accurate evaluation of the activation of PAR_2 by Pg proteases, excluding the possible concurrently activation of other receptors. In addition, contrary to the other studies that used the recombinant enzyme gingipain RgpB, we stimulated PAR_2 with the bacterium itself. However, in spite of the fact that Pg produces at least eight different endopeptidases and a number of exopeptidases that belong to the cysteine-, serine- and metallo-classes of peptidades, it is well documented that the trypsin-like proteinases, named gingipains, are the main responsible for its proteolytic activity. Accordingly, the results from the present study showed that pretreatment of Pg samples with a potent trypsin inhibitor (SBTI) decreased $[Ca^{2+}]_i$ signal on PAR_2KNRK cells, and did not interfere with the subsequent response to SLIGRL-NH_2. It was also verified that after desensitization of the receptor by 2 subsequent treatments with trypsin, the addition of Pg samples did not lead to any calcium response in PAR_2KNRK cells.

Interestingly, our results provided evidence that Pg is able to release proteolytic activity upon infection, as fluid samples collected from Pg-injected subcutaneous chambers in mice showed an increased proteolytic activity, which was

significantly inhibited by the addition of a SBTI. Moreover, it was shown by our results that this proteolytic activity was able to activate PAR_2, as fluid samples collected from Pg-treated mice provoked a calcium signal in KNRK cells transfected with PAR_2 but not in non-transfected cells or in cells that were desensitized by pre-exposure to trypsin.

A number of studies have suggested a dual role of PAR_2 in the inflammatory processs. While PAR_2 activation seems to be involved in leukocyte migration, inflammation of joints, skin, and kidney and allergic inflammation of airways (19), it has also been linked to some protective actions, suggesting an anti-inflammatory role for PAR_2 via the epithelium and vascular endothelium in the airways, in the mucosal tissues of the gastrointestinal tract, or in response to cardiovascular injury. Conversely, the results from the present study clearly demonstrate that upon infection, PAR_2 plays a pro-inflammatory role.

The present study constitutes the first *in vivo* demonstration of the association between PAR_2 and a bacterial protease leading to an inflammatory disease. Analysis of the fluid samples collected from dorsal chambers showed that PAR_2-knockout mice infected with *P. gingivalis* have a decreased inflammatory response when compared to wild-type animals. Our data demonstrated that in the presence of *P. gingivalis*, PAR_2 lead to a significant increase in inflammatory cell infiltration, which was inhibited by SBTI, and significantly reduced in PAR_2-deficient ($PAR_2^{-/-}$) mice. In addition, increased levels of prostaglandin E_2, interferon-gamma, interleukin-6 and interleukin-1β were found in chambers of Pg-treated mice compared to control saline-treated mice. All these inflammatory

mediators, with the exception of interleukin-6, were either not increased or significantly inhibited in $PAR_2^{-/-}$ mice infected with Pg. Interestingly, no differences were found regarding the interleukin-6 levels released upon Pg infection in $PAR_2^{-/-}$ mice compared to wild-type mice, thus suggesting that IL-6 production is not mediated through PAR_2 activation. On the other hand, Loubarkos et al, 2001 (8), have shown that in the oral epithelial cell line, which expresses both PAR_1 and PAR_2, treatment with gingipain-R induced secretion of IL-6. Thus, it could be suggested that in their study, the increased levels of IL-6 found after gingipain-R treatment were the result of PAR_1 activation, and not PAR_2.

The present study also provided an important role for PAR_2 in the alveolar bone loss of mice orally challenged with Pg, as a significant prevention of alveolar bone loss was verified in $PAR_2^{-/-}$ mice at 42 and 60 days after infection. Taken together, the results from the present study clearly show that PAR_2 is activated upon Pg infection, where it plays an important role in the host inflammatory response.

PAR_2 mediated activation of cells by bacterial proteases is an intriguing new mechanism in bacterial pathogenesis that remains to be clearly understood. Since PAR_2 is a one-shot receptor due to its desensitization after proteolysis, it can be considered as an emergency or sensor mechanism of the environment which is activated upon infection. However, its possible role in the maintenance of inflammation can not be overlooked since PAR_2 activation mediates the release of inflammatory mediators which, in theory, can up-regulate PAR_2

expression (20), therefore resulting in an ultimate increased severity of the disease.

High levels of proteolytic activity have been found in gingival crevicular fluid at periodontitis sites, where a mixture of endogenous host enzymes and bacterial proteases takes action leading to excessive degradation of connective tissue. Among these enzymes, neutrophil serine proteinase 3, mast cell tryptase, and gingipain have been isolated from the periodontal environment (reviewed in ref.21) and are believed to activate PAR_2. Therefore, it can be suggested that in a nearly future, therapies focused on the inhibition of bacterial proteinases or, more specifically, the use of PAR_2 antagonists could constitute an important approach for the modulation of an infectious pathology such as periodontal inflammatory disease.

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FIGURES

Figure 1:

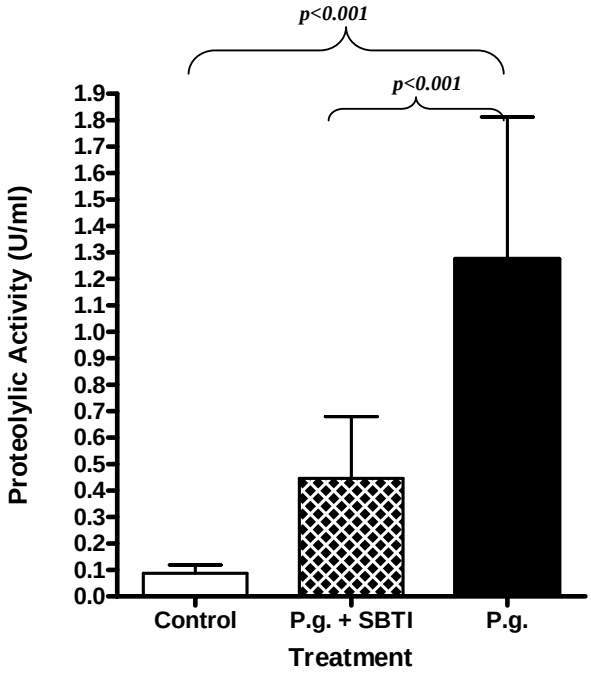
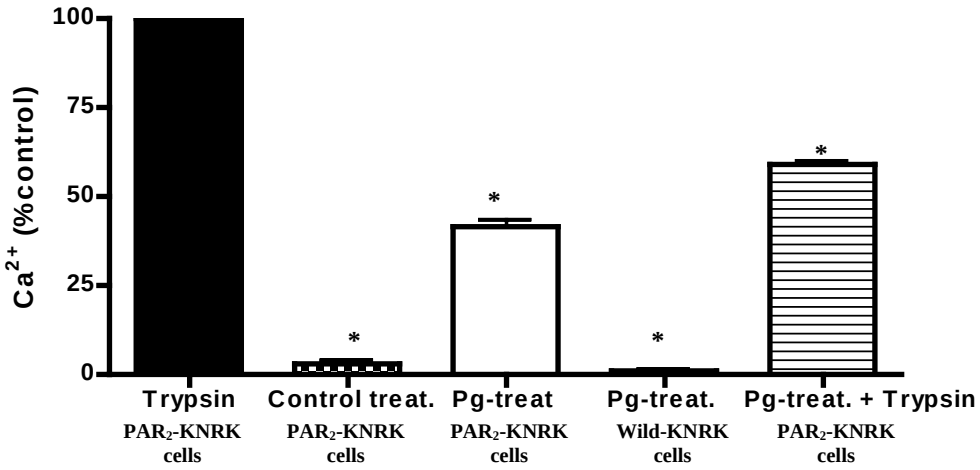


Figure 2

A


100
75
50
25
0
Ca2+ (%control)
*
*
*
*
Trypsin
PAR2-KNRK cells
Control treat.
PAR2-KNRK cells
Pg-treat
PAR2-KNRK cells
Pg-treat.
Wild-KNRK cells
Pg-treat. + Trypsin
PAR2-KNRK cells

B

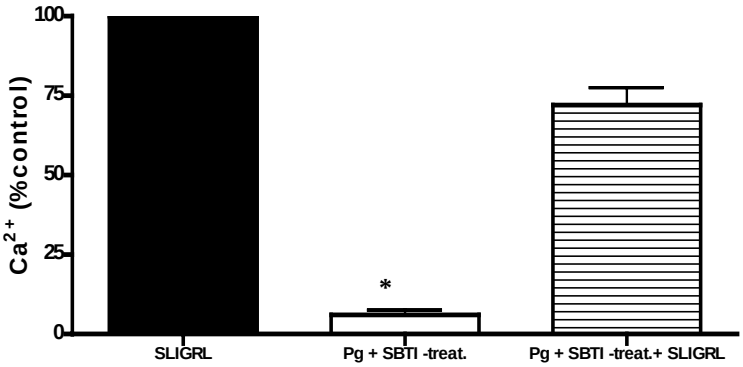

100
75
50
25
0
Ca2+ (%control)
*
SLIGRL
Pg + SBTI -treat.
Pg + SBTI -treat.+ SLIGRL

Figure 3

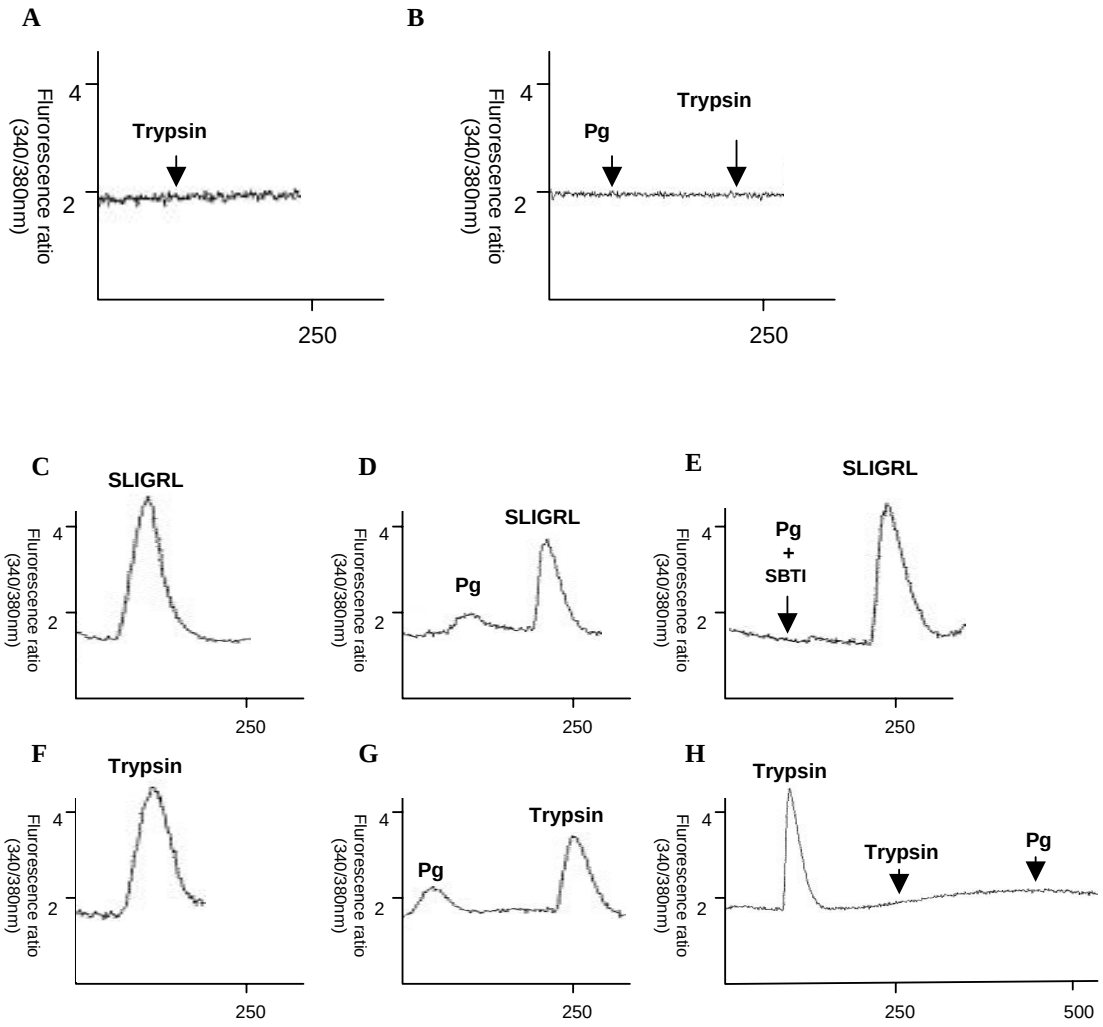

A
B
Flurorescence ratio (340/380nm)
4
2
Trypsin
Pg
250
C
D
E
SLIGRL
Pg + SBTI
F
G
H
500

Figure 4:

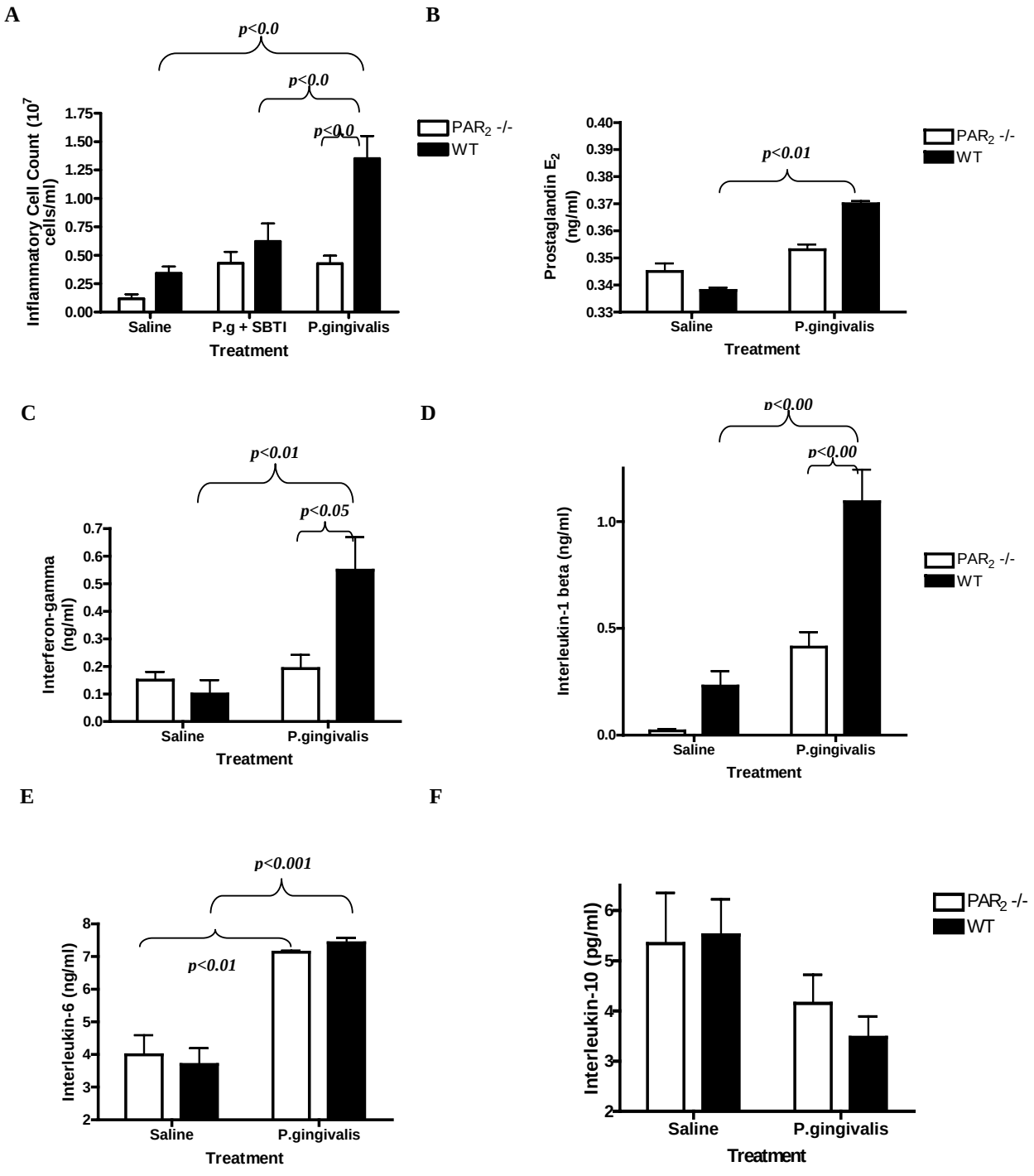
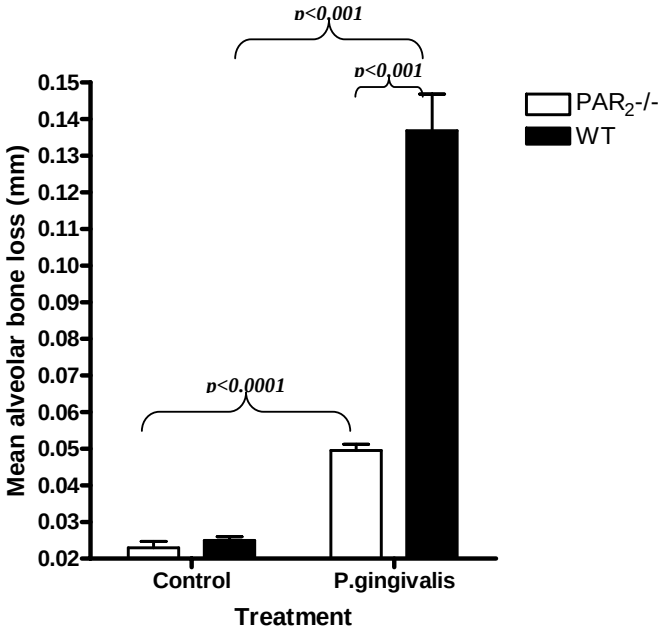

A
p<0.0
p<0.0
p<0.0
Inflammatory Cell Count (10^7 cells/ml)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Saline
P.g + SBTI
P.gingivalis
Treatment
PAR2 -/-
WT
B
Prostaglandin E2 (ng/ml)
0.40
0.39
0.38
0.37
0.36
0.35
0.34
0.33
p<0.01
Saline
P.gingivalis
Treatment
PAR2 -/-
WT
C
p<0.01
p<0.05
Interferon-gamma (ng/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Saline
P.gingivalis
Treatment
D
p<0.00
p<0.00
Interleukin-1 beta (ng/ml)
1.0
0.5
0.0
Saline
P.gingivalis
Treatment
PAR2 -/-
WT
E
p<0.001
p<0.01
Interleukin-6 (ng/ml)
8
7
6
5
4
3
2
Saline
P.gingivalis
Treatment
F
Interleukin-10 (pg/ml)
6
5
4
3
2
Saline
P.gingivalis
Treatment
PAR2 -/-
WT

Figure 5

A


p<0,001
p<0,001
p<0,0001
PAR2-/-
WT
Mean alveolar bone loss (mm)
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
Control
P.gingivalis
Treatment

B

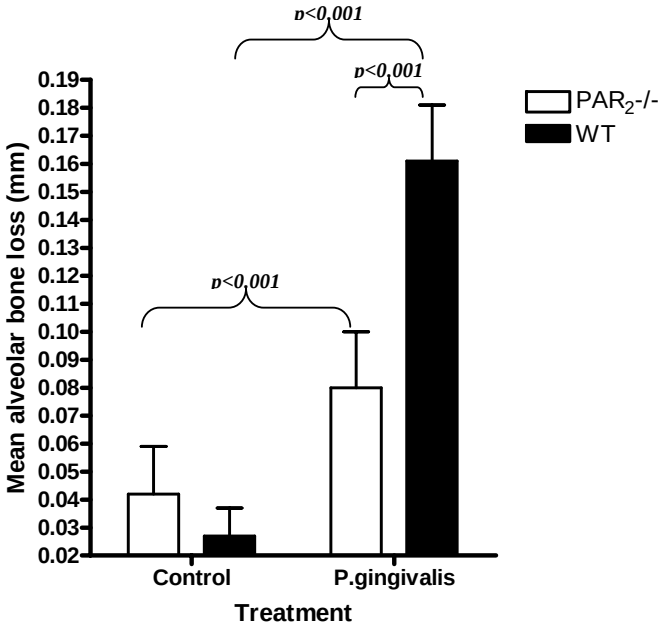

p<0,001
p<0,001
p<0,001
PAR2-/-
WT
Mean alveolar bone loss (mm)
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
Control
P.gingivalis
Treatment

FIGURE LEGENDS

Figure1. Proteolytic activity in fluid samples from WT mice. Samples from *Porphyromonas gingivalis* infected mice showed significantly higher ($p<0.05$) proteolytic activity when compared to control group. SBTI decreased the proteolytic activity due to *P. gingivalis* treatment. Means $\pm$ S.E.M., n = 10 (group).

Figure 2

A. Effect of fluid samples from *P. gingivalis* treated animals on intracellular calcium ion concentration $[Ca^{2+}]_i$ in PAR_2-expressing KNRK and wild-type KNRK cells. Chamber fluid samples (40µl) collected from WT mice after *P. gingivalis* infection decreased the subsequent calcium response by trypsin in KNRK cells expressing rat PAR_2. No $[Ca^{2+}]_i$ responses were verified with the addition of fluid samples collected from control animals. In addition, non-transfected KNRK cells did not respond to the addition of fluid samples from *P. gingivalis* infected animals. Means $\pm$ S.E.M. from eight separate experiments. * significant difference ($p<0.05$) *versus* Trypsin.

B. Effect of fluid samples from *P. gingivalis* and SBTI - treated animals on the calcium response in PAR_2-expressing KNRK cells. Fluid samples collected from WT mice treated with both *P. gingivalis* and soybean trypsin inhibitor (SBTI) lead to a minimal response, and decreased the subsequent calcium response by SLIGRL-NH_2 (Fig.**2B**). Means $\pm$ S.E.M. from eight separate experiments. * significant difference ($p<0.05$) *versus* Trypsin.

Figure 3. Effect of *P. gingivalis* samples on the calcium response in wild-type KNRK cells (**A** and **B**), and in PAR_2-expressing KNRK cells (Figs **C**-**H**). Representative data from three experiments with comparable results. In the wild KNRK cell line, no responses were verified after addition of trypsin (**A**) or *P. gingivalis* culture supernatant samples (**B**). In KNRK cells expressing PAR_2, *P. gingivalis* culture supernatant samples (40µl) lead to Ca^{2+} mobilization, decreasing the subsequent responses to SLIGRL-NH_2 (**C** and **D**) or trypsin (**F** and **G**). *P. gingivalis* samples pretreated with SBTI for 10 min failed to increase $[Ca^{2+}]_i$, and did not interfere with the calcium response lead by the subsequent addition of SLIGRL-NH_2 (**E**). After desensitization of the receptor by 2 subsequent treatments with trypsin.for 5 min, the addition of *P. gingivalis* samples did not lead to any calcium response (**H**).

Figure 4. Inflammatory effects of subcutaneous chamber infection with Pg in WT and PAR_2-/- mice. Means $\pm$ S.E.M., n = 10 (group).

A. Subcutaneous challenge of WT mice with *P. gingivalis* led to significantly ($p<0.05$) increased inflammatory cell count when compared to saline treatment. WT mice showed significant increased ($p<0.05$) inflammatory cell counts compared to PAR_2-/- mice.

Effects of subcutaneous bacterial challenge on the concentration of inflammatory mediators: **B**. Increased PGE_2 levels were found in WT mice treated with *P. gingivalis* when compared to saline treatment. No significant differences were observed between WT mice compared to PAR_2-/- mice. **C**. Interferon-gamma (IFN-γ) levels were significantly increased in *P.gingivalis* - treated WT mice when

compared to saline- treated WT mice, and compared to *P.gingivalis* - treated PAR_2-/- mice. **D**. Significant IL-1β levels were found in WT mice treated with *P. gingivalis* when compared to saline- treated WT mice, and compared to *P. gingivalis* - treated PAR_2-/- mice. **E**. *P. gingivalis* treatment led to significant ($p<0.05$) increases in the IL-6 levels when compared to saline treatment in WT and PAR_2-/- mice. **F**. There were no significant differences between the saline and *P. gingivalis* treated WT and PAR_2-/- mice regarding the IL-10 levels.

Figure 5.

PAR_2 activation role in *P. gingivalis* induced periodontitis. Significant increases in mean bone loss compared to saline-treated animals in WT (**A**) and PAR_2-/- (**B**) groups. WT mice showed significant higher ($p<0.05$) mean alveolar bone loss compared to PAR_2-/-. Means $\pm$ S.E.M., n = 10 (group).

DISCUSSÃO GERAL

O presente estudo demonstrou claramente que a ativação do receptor PAR_2 participa da regulação do processo inflamatório nos tecidos periodontais. Primeiramente, demonstrou-se que a ativação específica do PAR_2 é capaz de reproduzir aspectos envolvidos na periodontite (capítulo 1). Isto foi demonstrado através da aplicação tópica de um agonista seletivo do receptor PAR_2 em gengiva de ratos, o que induziu inflamação gengival e perda óssea alveolar. Além disso, demonstrou-se que a ativação seletiva do receptor PAR_2 leva à exacerbação de uma periodontite pre-existente, induzida pela colocação de ligadura. Ainda, demonstrou-se que a periodontite induzida pela aplicação tópica do agonista do PAR_2 é mediada por um mecanismo envolvendo a liberação de prostaglandinas e a ativação de metaloproteinases da matriz (MMP), especificamente MMP-2 e MMP-9. Desta forma, os resultados obtidos neste primeiro projeto sugeriram um importante papel do receptor PAR_2 na patologia da doença periodontal.

Sabe-se que o fluido crevicular gengival presente em sítios periodontais ativos apresenta altos níveis de atividade proteolítica proveniente de uma combinação de proteases endógenas e bacterianas, as quais podem levar à destruição dos tecidos periodontais[16] O mecanismo exato pelo qual estas proteases regulam a doença periodontal, ainda encontra-se longe de ser compreendido em detalhes. Baseado no fato de que gingipain, uma protease liberada por *P. gingivalis*,

mostrou-se capaz de ativar o receptor PAR_2 *in vitro* [26] e aliado aos resultados obtidos no primeiro projeto, sugeriu-se uma possível participação do PAR_2 na destruição periodontal por proteases bacterianas. Desta forma, com o intuito de complementar o projeto inicial, iniciou-se um estudo visando a demonstração do papel da ativação do receptor PAR_2 pela bactéria *Porphyromonas gingivalis* no desenvolvimento de resposta inflamatória no hospedeiro (capítulo 2). Estes experimentos foram realizados com camundongos geneticamente modificados, deficientes em PAR_2, tendo em vista a ausência de um antagonista específico para este receptor. Primeiramente demonstrou-se que amostras de fluido coletadas de câmera subcutânea implantada na região dorsal de camundongos e infectadas por *Porphyromonas gingivalis*, apresentaram uma alta atividade proteolítica a qual levou à ativação do PAR_2 em células KNRK transfectadas com o receptor PAR_2. Ainda, constatou-se que amostras da bactéria *Porphyromonas gingivalis* eram capazes de levar à ativação do PAR_2 em células KNRK transfectadas com o receptor PAR_2, desta forma descartando-se a possibilidade de que a atividade proteolítica proveniente não era apenas fruto de proteases produzidas pelo hospedeiro em decorrência da infecção, tais como a tripsina, a qual pode ser liberada por células endoteliais danificadas, ou triptase, a qual pode ser liberada sob degranulação de mastócitos.

Em seguida, avaliou-se o papel da ativação do PAR_2 na resposta inflamatória seguida à infecção por *Porphyromonas gingivalis* em câmeras subcutâneas implantadas em camundongos. A análise dos resultados obtidos demonstrou que o receptor PAR_2 foi responsável pelo recrutamento de células inflamatórias e

aumento de produção de interleucinas e mediadores inflamatórios induzidos pela infecção por *Porphyromonas gingivalis*. Finalmente, investigou-se o papel da ativação do receptor PAR_2 no desenvolvimento de doença periodontal induzida por *Porphyromonas gingivalis*. Os resultados demonstraram que a ativação do receptor PAR_2 desempenha um papel crucial na perda óssea alveolar induzida por *Porphyromonas gingivalis*. Desta forma, os resultados obtidos neste segundo projeto demonstraram claramente que o PAR_2 pode ser ativado na presença de infecção por Pg, exercendo um papel importante na resposta inflamatória do hospedeiro.

A participação do PAR_2 na regulação da resposta do hospedeiro por proteases bacterianas pode constituir-se em um novo e intrigante mecanismo na patogênese da infecção bacteriana. A princípio, pelo fato de o PAR_2 sofrer desensibilização após sua ativação por proteólise, ele pode ser considerado um mecanismo de emergência ativado pela infecção. Entretanto, o seu possível envolvimento na manutenção da inflamação, também deve ser considerado, uma vez que o PAR_2 regula a liberação de mediadores inflamatórios os quais, por sua vez, podem levar ao aumento de sua expressão nos tecidos, resultando no aumento da severidade da doença.

CONCLUSÕES GERAIS

Diante dos objetivos propostos pelo presente trabalho de tese, concluímos que:

- A aplicação tópica de um de agonista do PAR_2 (SLIGRL) causa periodontite em ratos, levando ao aumento significativo de perda óssea alveolar e de inflamação gengival.
- A aplicação tópica de SLIGRL exacerba periodontite pré-existente em ratos, levando ao aumento significativo de perda óssea alveolar e de infiltração granulocítica gengival.
- A inibição de MMP e de COX diminuiu a severidade da periodontite induzida por SLIGRL sugerindo o envolvimento destas enzimas no mecanismo pelo qual a ativação do PAR_2 leva à inflamação e perda óssea. Mais especificamente, ocorre um aumento da expressão tecidual da Cox-1, Cox-2, MMP-2 e MMP-9 após aplicação tópica gengival do agonista seletivo do PAR_2;
- A infecção por *Porphyromonas gingivalis* leva ao aumento da atividade proteolítica encontrada em amostras de fluido coletadas de câmera subcutânea implantada na região dorsal de camundongos.
- A atividade proteolítica subsequente à infecção por *Porphyromonas gingivalis* leva à ativação do PAR_2 em células KNRK transfectadas com o receptor PAR_2.

- Amostras da bactéria *Porphyromonas gingivalis* levam à ativação do PAR_2 em células KNRK transfectadas com o receptor PAR_2.
- A ativação do PAR_2, seguida à infecção por *Porphyromonas gingivalis* em câmeras subcutâneas implantadas em camundongos, leva ao aumento da resposta inflamatória caracterizada por maiores concentrações de células inflamatórias, prostaglandina E_2, interferon-gamma e interleucina-1β.
- A ativação do PAR_2, seguida à infecção oral por *Porphyromonas gingivalis*, leva ao aumento da perda óssea alveolar em camundongos aos 42 e 60 dias após infecção.

Desta forma, o receptor PAR_2 desempenha um importante papel na doença periodontal, e pode ser considerado um novo mecanismo na patogênese da infecção bacteriana. Em virtude disso, a inibição da ativação do PAR_2 poderá representar em um futuro próximo uma nova alternativa terapêutica na modulação da resposta do hospedeiro na inflamação periodontal.

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RESUMO

O receptor tipo 2 ativado por protease (PAR_2) é responsável pela regulação *in vitro* de respostas celulares a proteases, como a gingipain, que é produzida pelo periodontopatógeno *Porphyromonas gingivalis* (Pg). A principal hipótese do presente estudo foi a de que a ativação do PAR_2 participa da doença periodontal. Primeiramente, avaliou-se o papel da ativação do PAR_2, com o uso de uma agonista seletivo (SLIGRL), sobre a doença periodontal em ratos. Os resultados demonstraram que a aplicação tópica gengival de SLIGRL não apenas causou periodontite, mas também levou à exacerbação de uma periodontite existente, através de um mecanismo envolvendo a participação de cicloxigenases e metaloproteinases da matriz. Depois, avaliou-se a contribuição específica do PAR_2 na defesa do hospedeiro durante a infecção por Pg. A injeção de Pg em câmeras subcutâneas em camundongos levou ao aumento da atividade proteolítica, a qual foi responsável pela ativação do PAR_2 em células KNRK transfectadas com o receptor. Além disso, a inoculação de Pg induziu uma maior resposta inflamatória em camundongos normais comparada aos deficientes em PAR_2 ($PAR_2^{-/-}$). Ainda, a infecção oral com Pg resultou em perda óssea alveolar a qual foi reduzida em animais $PAR_2^{-/-}$ aos 42 e 60 dias após infecção. Os resultados do presente estudo demonstraram que o PAR_2 desempenha um importante papel na resposta inflamatória associada com a doença periodontal. No futuro, a inibição do PAR_2 poderá representar uma nova alternativa terapêutica na modulação da resposta do hospedeiro na periodontite.

ABSTRACT

Protease-Activated Receptor-2 (PAR_2) mediates *in vitro* cellular responses to proteases, such as gingipain, a protease produced by the periodontal pathogen *Porphyromonas gingivalis* (Pg). The main hypothesis of the present study was that PAR_2 activation may participate to periodontal disease. First, we have investigated the role of PAR_2 activation by a selective agonist (SLIGRL) on periodontal disease in rats. The results showed that gingival topical application of SLIGRL not only causes periodontitis but also exacerbates existing periodontitis through a mechanism involving activation of cyclooxigenases and matrix metalloproteinases.

Then, we have investigated the specific contribution of PAR_2 to host defense during Pg infection. Injection of Pg into subcutaneous chambers in mice resulted in increased proteolytic activity, which was able to activate PAR_2 in KNRK cells transfected with the receptor. In addition, Pg inoculation induced a higher inflammatory response in wild-type mice compared to PAR_2-deficient ($PAR_2^{-/-}$) mice. Moreover, oral infection with Pg resulted in alveolar bone loss which was significantly reduced in $PAR_2^{-/-}$ mice at 42 and 60 days after infection. Taken together, the results from the present study clearly show that PAR_2 plays an important role in the inflammatory response associated with periodontal disease. Inhibition of PAR_2 may represent in the future, a novel therapeutic approach that modulates the host response in periodontitis.

ANEXO 1

Role of Protease-Activated Receptor-2 (PAR_2) in inflammation, and its possible implications as a putative mediator of periodontitis

Memórias do Instituto Osvaldo cruz, aceito em novembro de 2004.

Role of Protease-Activated Receptor-2 (PAR_2) in inflammation, and its possible implications as a putative mediator of periodontitis

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Key Words: Protease-activated receptor-2; alveolar bone loss; inflammation; host response, *Porphyromonas gingivalis*, periodontitis.

Summary

Protease-activated receptor-2 (PAR_2) belongs to a novel subfamily of G-protein-coupled receptors with seven-transmembrane domains. This receptor is widely distributed throughout the body and seems to be importantly involved in inflammatory processes. PAR_2 can be activated by serine proteases such as trypsin, mast cell tryptase, and bacterial proteases, such as gingipain produced by *Porphyromonas gingivalis*. This review describes the current stage of knowledge of the possible mechanisms that link PAR_2 activation with periodontal disease, and proposes future therapeutic strategies to modulate the host response in the treatment of periodontitis.

Introduction

Protease-activated receptors (PARs) belong to a recently described family of G-protein-coupled, seven-transmembrane-domain receptors. Activation of PARs occurs through proteolytic cleavage of their N-terminal domain by proteases, resulting in the generation of a new N-terminal “tethered ligand”, which can autoactivate the receptor finction (Ossovskaya & Bunnett, 2004). Four members of the PAR family have been cloned. PAR_1, PAR_3, and PAR_4 can be activated by thrombin, and PAR_2 can be activated by trypsin, mast cell tryptase, neutrophil protease 3, tissue factor/ factor VIIa/ factor Xa, membrane-tethered serine protease-1, or proteases from *Porphyromonas gingivalis* (Vergnolle et al. 2001, Lourbakos *et al*., 2001).

Selective synthetic peptides, corresponding to the tethered ligand sequences, are able to activate selectively the receptors through direct binding to

the body of the receptor, without the need of proteolysis (Cocks & Moffatt, 2000). With the exception of PAR_3, all the other receptors have their selective agonist peptides. PAR_1, PAR_2, and PAR_4 can be non-enzimatically and selectively activated by TFLLR-NH_2, SLIGRL-NH_2, and GYPGQV-NH_2, respectively (Ossovskaya & Bunnett, 2004).

In spite of showing similar structures and common mechanisms of activation, the PARs have different tissue localization and function. PAR_1 can be found in human platelets, endothelium, epithelium, fibroblasts, myocytes, neurons, and astrocytes, and it seems to play a role in the vascular matrix deposition after injury. PAR_3 and PAR_4 are found in platelets, endothelium, myocytes, and astrocytes, and they are thought to be involved in the thrombus formation and pulmonary embolism (Ossovskaya & Bunnett, 2004). PAR_2 is found throughout the body, especially in the epithelium, endothelium, fibroblasts, osteoblasts, neutrophils, myocytes, neurons, and astrocytes (Abraham et al. 2000, Uehara et al. 2003, Ossovskaya & Bunnett, 2004). PAR_2 seems to play critical pathophysiological roles, as it is involved in leukocyte migration, inflammation of joints, skin, and kidney and allergic inflammation of airways (Ossovskaya & Bunnett, 2004)

PAR_2 and inflammation

The involvement of PAR_2 in inflammation is supported by several studies. Early studies reported that PAR_2 expression was up-regulated by inflammatory mediators such as tumour necrosis factor alfa, interleukin 1beta and lipopolysaccharide (Nystedt et al., 1996). Furthermore, deletion of PAR_2 also

diminishes inflammation in the airway and joints (Schmidlin et al. 2002, Ferrell et al. 2003), and delays the onset of inflammation (Lindner et al. 2000). Moreover, a number of studies have demonstrated that activation of PAR_2 can lead to blood vessel relaxation, hypotension, increased vascular permeability, granulocyte infiltration, leukocyte adhesion and margination, and pain (Cocks & Moffatt 2000, Vergnolle et al. 2001, Coughlin & Camerer 2003), all effects that encounter for the cardinal signs of inflammation. PAR_2 activation also leads to the release of prostanoids and cytokines including interleukin IL-6 and IL-8 in epithelial or non-epithelial cells (Lourbakos et al. 2001, Uehara et al. 2003). In the gastrointestinal tract, PAR_2 has been localized in many different cell types: in enterocytes, in endothelial cells of the lamina propria and the submucosa, in fibroblasts, in myenteric neurons, in immune and inflammatory cells (lymphocytes, neutrophils, mast cells) (Bohm et al. 1996, Nystedt et al. 1996). Recently, we have shown that in the colon, PAR_2 agonists (PAR_2-activating peptide, trypsin, tryptase) lead to an inflammatory reaction characterized by edema, granulocyte infiltration, increased intestinal permeability and pro-inflammatory cytokines (interleukin-1, TNF-alpha) release (Cenac et al. 2002). Recent studies also indicate an important role of PAR_2 in inflammatory pain. The receptor identified on sensory afferent nerves has been associated with long-lasting thermal and mechanical hyperalgesia in the soma as well as in visceral organs (Vergnolle N. Bunnett N.W. et al. 2001, Coelho et al. 2002).

Taken together, these studies suggest a pro-inflammatory role for PAR_2 *in vivo*, as it may mediate responses to tissue injury. These findings suggest that PAR_2 plays a crucial role in the regulation of inflammation.

Role of PAR_2 in periodontitis

A possible participation of PAR_2 in chronic oral inflammation such as periodontitis was indirectly suggested by several studies. First, gingipain, a bacterial protease produced by *Porphyromonas gingivalis*, a major causative agent of adult periodontitis, was reported to activate PAR_2 (Lourbakos et al. 2001). In addition, PAR_2 expression was found in osteoblasts, oral epithelial cells, and human gingival fibroblasts (Abraham et al. 2000, Lourbakos et al. 2001, Uehara et al. 2003). Lourbakos et al. (2001) showed that in an oral epithelial cell line, PAR_2 activation by purified gingipain induced the secretion of the pro-inflammatory cytokine interleukin-6 (IL-6), which is a potent stimulator of osteoclast differentiation and bone resorption. Uehara et al. (2003), demonstrated that a synthetic PAR_2 agonist peptide activates human gingival fibroblasts to produce IL-8 and to selectively stimulate MMP activity from these cells. This particular study suggests that PAR_2 activation could account for collagen destruction associated with periodontitis lesions. Most recently, a study by Chung et al. (2004), showed that PAR_2 is involved in the up-regulation of human beta-defensin in human gingival epithelial cells, stimulated by the peptide agonist of PAR_2, and *Porphyromonas gingivalis* proteases. Thus, this study points to a possible role for PAR_2 in the gingival tissues, where its activation could act as an emergengy mechanisms, that would constitute a first alarm in

mucosal tissues, alerting for the invasion of bacterial pathogens, and organizing a primary inflammatory response.

Taken together, these studies suggest a role for PAR_2 activation in inducing inflammation and bone resorption during periodontitis. However, another study by Smith *et al*. (2004) suggests that PAR_2 activation could inhibit bone resorption. In that study, the authors showed that the selective PAR_2-activating peptide SLIGRL-NH_2 inhibited osteoclast differentiation, thereby acting as a potential inhibitor of bone destruction. This result which contradicts the suggested role for PAR_2 activation in bone loss, reflects the difficulties of using *in vitro* approaches to evaluate the role of the different mediators that are involved in periodontal diseases.

The experiments from our group (data not published) provided the first evidences for *in vivo* evaluation of the role of PAR_2 activation in periodontitis. We showed that local application of a selective PAR_2 agonist (SLIGRL) in oral cavity of rats, causes gingival granulocyte infiltration, and periodontitis through a mechanism involving prostaglandin release and matrix metalloproteinase activation. In addition, seven days after PAR_2-agonist treatment, a peak of granulocyte infiltration (measured by an increased myeloperoxydase (MPO) activity) was observed. As polymorphonuclear neutrophils represent the main source for MPO in acute inflammation, and because they constitute the frontline of the acute host inflammatory response, promoting the release of a number of inflammatory mediators that are able to stimulate osteoclasts (Dennison & Van Dyke, 1997), it can be proposed that recruited neutrophils might be responsible,

at least in part, for the initiation of periodontitis. Therefore, our study also suggests that PAR_2 agonist-induced bone loss is due, at least in part, to the induction of an acute inflammatory response. In agreement with previous *in vitro* studies which supported a destructive role for PAR_2 (Lourbakos et al. 2001; Uehara et al. 2000), our *in vivo* approach definitively demonstrated a pro-inflammatory and bone destruction role for PAR_2 activation in periodontal tissues.

Interestingly, gingipains-R (RgpB and HRgpA) activate also the protease-activated receptors, PAR_1 and PAR_4, which are expressed on the surface of platelets and are responsible for platelet aggregation (Lourbakos A. Yuan Y. et al, 2001). This mechanism may constitute the biological plausibility of the association between periodontitis and cardiovascular disease.

Conclusions

The pro-inflammatory role of PAR_2 in inflammation is adequately and clearly demonstrated by several studies, which showed that PAR_2 activation leads to widespread pro-inflammatory effects, including the release of pro-inflammatory cytokines, and regulation of a number of inflammatory diseases.

The association of PAR_2 with the pathogenesis of periodontitis is supported by some concepts:

i) PAR_2 can be activated by gingipain, a bacterial protease produced by the major periodontopathogen, *Porphyromonas gingivalis*;

ii) PAR_2 is expressed by cells that are actively involved in periodontal pathologies, such as oral epithelial cells, fibroblasts, and osteoblasts,

and PAR_2 activation in those cells leads to the production of mediators of bone resorption;

iii) PAR_2 activation by a selective peptide agonist leads to gingival granulocyte infiltration, and alveolar bone loss in rats, through a mechanism involving prostaglandin release and matrix metalloproteinase activation.

These findings indicate that PAR_2 might represent a potential target for the design of drug therapies focused on the modulation of periodontal inflammation.

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