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EFEITO MODULATÓRIO DE FATORES DE VIRULÊNCIA DE *Porphyromonas gingivalis* SOBRE OS RECEPTORES B_1 PARA AS CININAS NA PATA DE RATO

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Dissertação apresentada como requisito para obtenção do título de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul

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Co-Orientador: Prof. Dr. Eraldo L. Batista Jr.

PORTO ALEGRE, 2009

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"Deus não escolhe os capacitados
capacita os escolhidos
Fazer ou não fazer algo
só depende de nossa vontade
e perseverança."
Albert Einstein

RESUMO

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O receptor B_1 para as cininas está normalmente ausente durante condições fisiológicas podendo ser rapidamente induzido em resposta a diversos estímulos, como os processos infecciosos. Apesar disso, não existem estudos indicando como o LPS de *Porphyromonas gingivalis* (Pg-LPS), um ativador preferencial de TLR2, pode induzir a expressão do receptor B_1. No presente trabalho, foi demonstrado que a injeção do Pg-LPS na pata de rato resulta na indução funcional tempo-dependente dos receptores B_1 (determinada pelo aumento marcante na resposta edematogênica), que foi precedida pelo rápido aumento na expressão do seu RNAm. A administração local de Pg-LPS produziu um aumento marcante na produção da citocina pró-inflamatória TNF-α e do influxo de neutrófilos. Ambos os eventos foram observados anteriormente à indução dos receptores B_1. A indução funcional e molecular do receptor B_1 pelo Pg-LPS foi reduzida significantemente pelo glicocorticóide dexametasona e pelo anticorpo monoclonal anti-TNF-α infliximab. De grande relevância, foi demonstrado pela primeira vez, que uma única administração de Resolvina E1, um mediador lipídico pró-resolução da inflamação, foi capaz de produzir uma redução marcante na expressão dos receptores B_1 mediada pelo Pg-LPS, provavelmente inibindo produção de TNF-α e a migração de neutrófilos para o sítio inflamatório. Analisados em conjunto, os dados obtidos permitem inferir que a ativação do TLR2 pelo Pg-LPS é capaz de induzir a expressão dos receptores B_1, através de mecanismos relacionados à produção de TNF-α e ao influxo de neutrófilos, que são sensíveis a Resolvina E1. Assim, é possível sugerir que os receptores B_1 representam uma via importante nas respostas inflamatórias desencadeadas pela *P. gingivalis* e seus fatores de virulência.

ABSTRACT

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It has been demonstrated that kinin B_1 receptors are highly upregulated under several stressful stimuli, such as infection. In spite of that, there is no evidence indicating whether *Porphyromonas gingivalis* LPS (Pg-LPS), a preferential TLR2 activator, might lead to B_1 receptor upregulation. In this study, we demonstrate that Pg-LPS injection into the rat paw resulted in a marked functional upregulation of B_1 receptors (as measured by an increase of B_1 receptor-induced edema formation), which was preceded by a rapid raise in B_1 receptor mRNA expression. The local administration of Pg-LPS also resulted in a prominent production of the proinflammatory cytokine TNF-α, followed by an increase of neutrophil influx; both events were observed at time periods prior to B_1 receptor induction. The functional and molecular Pg-LPS-elicited B_1 receptor upregulation was significantly reduced by the glucocorticoid dexamethasone, and to a lesser extent by the chimeric anti-TNFα antibody infliximab. Of high relevance, we show for the first time that a single administration of the pro-resolution lipid mediator Resolvin E1 was able to markedly down-regulate Pg-LPS-driven B_1 receptor expression, probably by inhibiting TNFα production and neutrophil migration to the inflammatory set. Collectively, the present findings clearly suggest that TLR2 activation by Pg-LPS is able to induce the upregulation of B_1 receptors, through mechanisms involving TNFα release and neutrophil influx, which are largely sensitive to Resolvin E1. It is tempting to suggest that kinin B_1 receptors might well represent a pivotal pathway for the inflammatory responses evoked by *P. gingivalis* and its virulence factors.

SUMÁRIO

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INTRODUÇÃO

1. INTRODUÇÃO

Recentemente, o processo inflamatório tem sido apontado como um fator etiológico de muitas doenças, incluindo algumas nunca antes associadas à inflamação, como doença de Alzheimer, doenças cardiovasculares e câncer. Tem-se tornado cada vez mais evidente a similaridade dessas patologias, com doenças de origem tipicamente inflamatória, como a artrite reumatóide e a periodontite (Van Dyke, 2007). Desta forma, a caracterização dos mecanismos envolvidos na resposta inflamatória tem sido alvo de muitos estudos e revisões recentes (Weiss, 2008).

O processo inflamatório pode ser definido como um conjunto de alterações bioquímicas e celulares que ocorre nos tecidos, em resposta a estímulos como trauma e infecção. A inflamação é caracterizada pelo aumento do fluxo sanguíneo no local afetado, aumento da permeabilidade vascular e migração de leucócitos para o sítio inflamatório. Esse influxo celular é regulado por mediadores inflamatórios produzidos por células endoteliais e inflamatórias. Os mediadores inflamatórios são moléculas solúveis e compreendem os produtos da degranulação dos mastócitos (histamina e serotonina), os peptídeos vasoativos (cininas, neurocininas e peptídeo relacionado ao gene da calcitonina), os componentes do sistema complemento, os mediadores lipídicos (prostaglandinas, leucotrienos e PAF), as citocinas, quimiocinas e as enzimas proteolíticas (Nathan, 2002; Medzhitov, 2008), entre outros.

As cininas são peptídeos biologicamente ativos que participam da resposta inflamatória, promovendo vasodilatação, aumento da permeabilidade vascular, extravasamento plasmático e migração celular (Marceau e Regoli, 2004; Hu *et al*., 2006;

Schulze-Thopoff *et al.,* 2008). Estão presentes em condições como sepse, dano pós-isquêmico, asma, pancreatite, cistite, alergia, diabetes, artrite reumatóide, colite, gastrite e câncer, além de causarem dor e hiperalgesia. As cininas também apresentam ações fisiológicas, participando do controle da pressão arterial, relaxamento e contração da musculatura lisa e natriurese (Calixto *et al.,* 2004; Marceau e Regoli, 2004; Stewart, 2004; Ferreira *et al*., 2005; Fox *et al*., 2005; Leeb-Lundberg *et al*., 2005; Wang *et al*., 2006; Schulze-Thopoff *et al.,* 2008).

O estudo do sistema das cininas teve inicio em 1909, quando Abelous e Bardier demonstraram que a injeção de uma fração insolúvel da urina humana, quando administrada endovenosamente em cães, era capaz de produzir uma queda acentuada na pressão sanguínea. Mais tarde, essas observações foram confirmadas por Pribram e Hernheiser (1920) e por Frey (1926), que apresentaram resultados semelhantes em animais de diferentes espécies. O efeito hipotensor observado foi atribuído a um componente isolado da urina humana, chamado inicialmente de substância F (Frey e Kraut, 1928). Posteriormente, Kraut e colaboradores (1930) encontraram quantidades elevadas de substância F no pâncreas, que foi indicado como o sítio principal de síntese da nova substância. A partir dessa descoberta, a substância F passou a se chamar calicreína (do grego: *kallikreas* relacionado ao pâncreas).

Em 1937, Werle e colaboradores demonstraram que a calicreína, quando incubada no plasma, era capaz de liberar uma potente substância contrátil a partir de um precursor inativo, denominada calidina. Mais tarde, Rocha e Silva e colaboradores (1949) observaram que a incubação do veneno da serpente *Bothrops jararaca,* ou de tripsina, com a fração pseudoglobulina do plasma era capaz de liberar um potente agente

vasodilatador e contracturante. Como essa substância produzia uma resposta contrátil lenta quando comparada com a histamina e a acetilcolina em preparações de íleo isolado de cobaia, foi denominada bradicinina (BK; do grego: *bradis*, para lento; *kinesia* para movimento). Em 1956, Andrade e Rocha e Silva purificaram a BK, mas apenas em 1960 a seqüência correta de aminoácidos foi determinada, quando Boissonas e colaboradores sintetizaram o nonapeptídeo: *Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg*. Com o advento da BK sintética tiveram inicio os primeiros estudos para a caracterização das ações biológicas desse peptídeo. Em 1964, Lewis demonstrou pela primeira vez a capacidade da BK em evocar os sinais clássicos da inflamação como aumento da permeabilidade vascular, formação de edema e dor. Mais tarde, em 1980, Regoli e Barabé descreveram as ações fisiológicas da bradicinina e seus análogos em diferentes tecidos. Atualmente, a BK e as cininas relacionadas têm sido implicadas em uma série de condições patofisiológicas (Regoli e Barabé, 1980; Marceau *et al*., 1998; Calixto *et al*., 2000; 2001; 2004; Marceau e Regoli, 2004; Bengtson *et al*., 2006; Costa-Neto *et al*., 2008).

A cascata de formação das cininas compreende mecanismos bem caracterizados. As cininas são formadas a partir de α-globulinas chamadas cininogênios. São conhecidos três tipos de cininogênios que diferem em tamanho, função e estrutura. O cininogênio de alto peso molecular (High Molecular Weight Kininogen, HMWK) é uma proteína plasmática com massa molecular de 120 kDa e dá origem à bradicinina (BK). O cininogênio de baixo peso molecular (Low Molecular Weight Kininogen, LMWK) tem massa molecular de 66 kDa e origina a calidina (Lys-BK), além da BK, estando amplamente distribuído nos tecidos, em fibroblastos e em outras estruturas celulares do

tecido conectivo. O terceiro tipo de cininogênio, tipo T, corresponde ao HMWK e é encontrado apenas em ratos (Bhoola *et al*.,1992; Mclean *et al*., 2000).

Os cininogênios são clivados por proteases chamadas calicreínas que são encontradas no sangue (calicreína plasmática) ou nas glândulas exócrinas (calicreína tecidual). A calicreína plasmática é produzida no fígado e circula na sua forma inativada, chamada de pré-calicreína ou fator de Fletcher. Após sua clivagem, dependente da ativação do fator de Hagemann (fator XII da coagulação sangüinea), é formada a enzima ativa. A calicreína plasmática age então sobre o HMWK liberando BK. Este processo está aumentado durante a resposta inflamatória (Mclean *et al.*, 2000).

A BK tem uma meia-vida plasmática muito curta, que varia entre 10 e 50 segundos. As cininases pertencem a um grupo de enzimas responsáveis pelo metabolismo e degradação das cininas. A cininase II, conhecida também como enzima conversora da angiotensina (ECA), é encontrada na membrana das células endoteliais e age sobre as cininas, removendo o dipeptídeo da porção C-terminal e originando metabólitos inativos. A endopeptidase neutra e a aminopeptidase plasmática também exercem um papel importante no metabolismo das cininas. A primeira está presente nas células epiteliais e utiliza um mecanismo semelhante ao da cininase II para inativar a BK (Gafford *et al*. 1983; Bhoola *et al*. 1992). Já a aminopeptidase é capaz de converter a Lys-BK em BK, através da clivagem da porção N-terminal (Guimarães *et al.* 1973). A cininase I, conhecida como arginina carboxipeptidase, é representada pela carboxipeptidase N (plasma) e carboxipeptidase M (membrana) e apresenta um papel menor na degradação da BK. Entretanto, essa enzima é responsável pela remoção da arginina da porção C-terminal da BK e da Lys-BK gerando os metabólitos ativos des-Arg^{9}-BK e Lys-des-Arg^{9}-

BK, respectivamente. A cininase II possui maior afinidade pela BK e pela Lys-BK do que a cininase I, o que sugere que a formação dos metabólitos ativos não ocorre sob condições fisiológicas. De fato, a formação desses metabólitos está presente em exsudatos inflamatórios, onde a formação de fibrina aumenta a atividade da cininase I em relação à cininase II (Campbell *et al*. 1990; Heindriks *et al*. 1990).

Depois de liberados, a BK e seus metabólitos podem ativar dois subtipos de receptores acoplados a proteína G, chamados de B_1 e B_2. A classificação dos receptores cininérgicos foi realizada inicialmente através de estudos farmacológicos no final da década de 70 (Regoli *et al*., 1977; Regoli e Barabé, 1980; Calixto *et al*., 2000; 2001; Leeb-Lundberg *et al*., 2005; Campos *et al*., 2006). Posteriormente, a existência dos receptores B_1 e B_2 foi confirmada por estudos de clonagem e de deleção gênica (Calixto *et al*., 2000; 2001; Leeb-Lundberg *et al*., 2005; Pesquero e Bader, 2006). Em conjunto, os estudos farmacológicos e de biologia molecular permitiram determinar as principais características dos receptores para as cininas, bem como as diferenças entre os dois subtipos de receptores.

Os receptores B_2 são expressos constitutivamente na maior parte dos tecidos e apresentam alta afinidade pela BK e pela Lys-BK. Já os receptores B_1 não são comumente expressos em condições normais, com exceção do sistema nervoso central, mas são rapidamente induzidos após estímulos como inflamação, infecção ou trauma e apresentam afinidade pelos metabólitos ativos des-Arg^9-BK e Lys-des-Arg^9-BK. Assim, tem sido sugerido que os receptores B_2 seriam responsáveis por mediar as respostas fisiológicas das cininas, mas também participariam da fase aguda dos processos inflamatórios. Por outro lado, os receptores B_1 seriam induzidos apenas em condições

patológicas, sendo envolvidos na amplificação e manutenção de alterações observadas nas respostas inflamatórias crônicas (Ni *et al*., 2003; Calixto *et al*., 2004; Marceau e Regoli, 2004; Stewart, 2004; Ferreira *et al*., 2005; Fox *et al*., 2005; Leeb-Lundberg *et al*., 2005; Wang *et al*., 2006; Hara *et al*., 2008). Esta idéia permite inferir que os receptores B_1 representam alvos de grande importância para o desenvolvimento de drogas com potencial antiinflamatório e que poderiam ser úteis para o tratamento de doenças inflamatórias crônicas como asma, artrite, osteoartrite, neuropatias e doença periodontal, entre outras (Campos et al., 2006; Kuduk e Bock, 2008).

A indução dos receptores B_1 tem sido associada com a produção de mediadores inflamatórios, com a ativação de células do sistema imune e, ainda, com a estimulação de diversas vias de sinalização intracelular. O gene que codifica o receptor B_1 para as cininas apresenta várias seqüências específicas para a ligação de fatores de transcrição. Uma série de evidências tem apontado o envolvimento do fator de transcrição NF-κB nos processos de regulação do receptor B_1 (Fernandes *et al.*, 2005). Ni e colaboradores (1998) demonstraram que mutações do sítio para ligação do NF-κB são capazes de abolir a indução do receptor em resposta a estímulos como LPS, IL-1β e TNF-α. O controle da expressão de receptores B_1 parece ocorrer em nível pós-transcripcional, através da modulação do seu RNAm (Zhou *et al*., 1999) Entretanto, esses mecanismos pós-transcricionais ainda são pouco caracterizados, necessitando, assim, da realização de estudos adicionais.

A doença periodontal ou periodontite tem alta prevalência no mundo e pertence a um grupo de infecções crônicas que causam inflamação dos tecidos gengivais e destruição dos tecidos periodontais, podendo, em casos mais graves, causar reabsorção

óssea e, conseqüentemente, perda de elementos dentários (Nishida *et al*., 2001; Bartold e Narayanan, 2006; Carayoll *et al*., 2006; Cochran, 2008). A doença é causada por um acúmulo de placa bacteriana na gengiva e ao redor do dente. Quando não tratada, pode se difundir para sítios de difícil acesso. Até o presente, mais de 300 espécies diferentes de bactérias já foram identificadas como causadoras da doença periodontal, mas a *Porphyromonas gingivalis*, uma bactéria gram-negativa, é considerada como tendo o maior potencial patogênico (Han *et al*, 2005). De grande relevância, vários estudos têm relacionado a doença periodontal com o desenvolvimento de doenças inflamatórias sistêmicas, como a aterosclerose e artrite reumatóide (Genco *et al*., 1998; Kinane e Lowe, 2000; Li *et al*., 2002; Rosenstein *et al*., 2004; Kantarci e Van Dyke, 2005; Madianos *et al*., 2005; Pihlstrom *et al*., 2005; Schenkein, 2006; Williams, 2008).

A *P. gingivalis* parece estar implicada tanto no início, quanto na progressão da doença periodontal. Embora a destruição tecidual seja causada parcialmente pelas proteases secretadas pela bactéria, acredita-se que a resposta imune do hospedeiro a esses produtos bacterianos é a maior causa de sua patogênese (Garlet *et al*., 2003; Zhou e Amar, 2006; Preshaw, 2008). Produtos da *P. gingivalis*, como o lipopolissacarídeo (LPS), as proteínas de membrana e as proteases bacterianas são capazes de induzir uma resposta celular local, através da secreção de inúmeras citocinas que levam à destruição do tecido periodontal (Carayol *et al*., 2006; Graves, 2008).

O LPS é um polímero que integra a superfície externa das bactérias gram-negativas e é capaz de recrutar diversos sistemas efetores intracelulares. A molécula de LPS é composta por um domínio hidrofóbico altamente conservado, chamado de lipídeo A ou endotoxina, além de apresentar um domínio hidrofílico polissacarídico (carboidrato)

de caráter variável. Essa porção polissacarídica do LPS é formada por um centro oligossacarídico e uma cadeia polissacarídica distal chamada antígeno "O". O lipídeo A é o responsável pela ação patogênica da bactéria e pela antigenicidade do LPS. Já o antígeno "O" está associado à resistência da bactéria aos antibióticos, ao sistema do complemento e ao estresse (Caroff *et al,* 2002; Raetz e Whitfield, 2002; Whitfield *et al*, 2003). De acordo com a dose, a via de administração e a sensibilidade individual, a administração de LPS pode induzir desde um aumento da resistência imune contra infecções até o aparecimento de sintomas patológicos mais severos, característicos do choque séptico (Alexander e Rietschel, 2001). A administração de doses elevadas de LPS de várias espécies de bactérias pode causar hipotensão, neutropenia, coagulação intravascular disseminada, produção de citocinas pró-inflamatórias e proteínas de fase aguda. Já, a injeção tecidual de pequenas quantidades de LPS é capaz de induzir a expressão de diferentes mediadores inflamatórios como quimiocinas, citocinas e moléculas de adesão, o que ocasiona recrutamento celular e a formação de edema (Ulevitch e Tobias, 1995; Saban *et al.,* 2001; Caroff *et al.,* 2002; Savard *et al.*, 2002).

A maior parte dos efeitos do LPS se deve à estimulação de uma família de receptores denominados receptores Toll-like (TLR). Em 1996, Lemaitre e colaboradores demonstraram que a proteína *toll*, presente em moscas da espécie *Drosophilla melanogaster*, além de estar envolvida no desenvolvimento embrionário, também era necessária para a geração de uma resposta imune contra fungos. Em 1997, uma proteína homóloga à *toll* foi clonada e caracterizada em humanos, sendo denominada de TLR (Medzhitov *et al.*, 1997; Rock *et al*., 1998; Akira *et al*., 2001). Os TLR são responsáveis pela identificação de padrões moleculares de diversos microorganismos como fungos,

bactérias, vírus e protozoários. Assim como na drosófila, o receptor *Toll* humano é uma proteína transmembrana do tipo I, com um domínio extracelular composto por regiões ricas em leucina e um domínio citoplasmático similar ao do receptor de IL-1. Após o reconhecimento do LPS, o receptor ativa algumas vias de sinalização intracelular relacionadas à proteína de diferenciação mielóide 88 (MyD88), à ativação da proteína quinase associada ao receptor de IL-1 (IRAK) e à associação de fatores relacionados ao receptor de TNF (TRAF-6) (Beutler, 2004; Ulevitch, 2004; Gerold *et al*, 2007). A ativação desses receptores pode resultar ainda na estimulação de proteínas da família das MAPKs (proteínas quinases ativadas por mitógeno) que estão envolvidas na regulação de fatores de transcrição como o NF-κB, responsável pela transcrição gênica (Medzhitov, 2001; Gay *et al.,* 2006). Dentre esses receptores, o TLR4 parece ser o responsável pelo reconhecimento do LPS de bactérias gram-negativas. Entretanto, estudos recentes demonstraram que o LPS de *P. gingivalis* parece ativar preferencialmente o TLR2 (Hirschfeld *et al*., 2001; Martin *et al*., 2001; Pulendran *et al*, 2001; Muthukuru *et al*, 2005; Hajishengallis *et al*, 2006; Kikkert *et al*, 2007).

Como mencionado anteriormente, processos infecciosos constituem estímulos clássicos para a indução dos receptores B_1 (Marceau *et al*., 1998; Calixto *et al*., 2000; 2001; 2004; Bengston *et al*., 2006). Por exemplo, um estudo conduzido por DeBlois e Horlick (2001), em macacos, indicou que o agonista seletivo dos receptores B_1, a des-Arg^9-BK, é capaz de induzir edema de pele, apenas em animais pré-tratados com LPS de *E. coli*. Além disso, foi demonstrado que o LPS de *E. coli* produz um aumento marcante do extravasamento plasmático induzido pela des-Arg^9-BK no duodeno, na traquéia e no íleo de ratos (Wille *et al*., 2001). Mais recentemente, Passos e colaboradores (2004)

mostraram a ocorrência de indução dos receptores B_1 após a administração de LPS de *E. coli* na pata de ratos, em um processo envolvendo a ativação do fator de transcrição NF-κB, a migração de neutrófilos, a liberação de citocinas como IL-1β e TNFα e o mediador lipídico PAF (fator de ativação plaquetária). Outros trabalhos recentes conduzidos *in vitro* mostraram que a incubação contínua com LPS de *E. coli* em preparações de traquéia de camundongo (Bachar *et al*., 2004), veia porta de rato (Medeiros *et al*., 2004) ou íris de porco (El Sayah *et al*., 2006) induz um aumento tempo-dependente das respostas contráteis mediadas pelos receptores B_1 para as cininas. Cabe destacar que as ações do LPS de *E. coli* estão associadas com a ativação de receptores TLR4 e com a subseqüente estimulação do fator nuclear NF-κB e de várias proteínas quinases, além da produção de citocinas pró-inflamatórias, sendo todos esses sinais relacionados com a indução dos receptores B_1 (Beg, 2002; Dobrovolskaia e Vogel, 2002; Akira *et al.,* 2003). Muito recentemente, Kikkert e colaboradores (2007) demonstraram que o LPS de *P. gingivalis* ativa preferencialmente TLR2, enquanto que o LPS de *E. coli* ativa TLR4. A ativação preferencial de receptores TLR2 também parece ocorrer em resposta à estimulação com *P. gingivalis* inativada por calor (Schindler *et al*., 1994; Yoshimura *et al*., 2002). Até o momento, ainda não há estudos indicando a relação entre a ativação de receptores TLR2 e a indução dos receptores B_1.

Tem sido demonstrado que a *P. gingivalis* é capaz de estimular células do sistema imune, levando ao aumento da produção de citocinas pró-inflamatórias tais como, IL-1β, TNF-α, IL-6 e IL-8, além de outros mediadores da inflamação, como PGE_2 (Kesavalu *et al.,* 2002; Bodet *et al.,* 2006; Eskan et al., 2008). Ademais, a *P. gingivalis* produz um grupo de cisteinil-proteases potentes, denominadas gingipaínas, que exercem um papel

fundamental na patogênese da doença periodontal (Imamura *et al.,* 1994; Kitano *et al*., 2001). Trabalhos anteriores mostraram que as gingipaínas são capazes de induzir a formação de BK, através da ativação de calicreínas ou, ainda, agindo diretamente sobre os cininogênios (Travis *et al.,* 1997; 2000; Imamura *et al.,* 2004). Desta forma, o aumento da permeabilidade vascular causado pela administração de proteases obtidas de *P. gingivalis* em cobaias foi significativamente aumentado pelo tratamento com inibidores da degradação de BK (Kaminishi *et al.,* 1993). Foi também demonstrado que a resposta inflamatória induzida por gingipaínas na bochecha de Hamsters pode ser prevenida pelo antagonista seletivo dos receptores B_2, NPC 17647 (Rubinstein *et al.,* 2001). Além disso, o aumento da permeabilidade vascular induzido pela injeção da cepa A7436 de *P. gingivalis* em camundongos foi potencializado pela BK ou por inibidores de sua degradação ou, ainda, reduzido por antagonistas dos receptores B_2 ou por cininases (Hu *et al*., 2006). Estes dados permitem sugerir que a BK e outras cininas poderiam apresentar um papel relevante na manutenção da doença periodontal. De fato, foi descrito que a BK, através da estimulação de receptores B_2, induz liberação de ácido araquidônico, com conseqüente aumento dos níveis de PGE_2 em fibroblastos gengivais humanos (Modéer *et al*., 1990). Além disso, foi demonstrado que a BK pode potencializar a produção de PGE_2 induzida por IL-1β ou a geração de IL-1β por TNFα nesta mesma linhagem de células (Lerner e Modéer, 1991; Yucel-Lindberg *et al.,* 1995). Tanto a BK, quanto o agonista seletivo de receptores B_1, des-Arg^9-BK, foram capazes de levar a um aumento da produção de IL-6, bem como de potencializar os efeitos da IL-1β e do TNFα sobre a formação desta citocina em fibroblastos gengivais humanos (Modéer *et al.,* 1998). Um efeito semelhante foi descrito para a produção de IL-8 após a incubação

de BK, através de mecanismos dependentes da ativação dos receptores B_2 e da estimulação de proteína quinase C e de MAP-quinases (Brunius *et al.,* 2005).

De forma interessante, um estudo conduzido por Griesbacher *et al.* (1994) mostrou que a injeção intraplantar de um extrato de *P. gingivalis* produz edema de pata e extravasamento plasmático, acompanhados de alterações nociceptivas na pata de rato. Estas respostas foram significativamente inibidas pelo tratamento sistêmico com o antagonista seletivo dos receptores B_2, Hoe 140, embora o papel dos receptores B_1 não tenha sido avaliado.

O modelo de edema de pata de rato tem sido bastante utilizado para investigar os mecanismos envolvidos nos processos de regulação dos receptores B_1 *in vivo*. Neste modelo, a injeção intraplantar do agonista seletivo dos receptores B_1, des-Arg^9-BK, produz apenas uma discreta alteração de volume em patas de animais controle. Por outro lado, a injeção deste agonista produz uma resposta edematogênica marcante após o tratamento local com vários agentes incluindo, IL-1β, TNFα, PAF ou LPS de *E. coli* (Campos *et al*., 1998; Fernandes *et al.,* 2003; Passos *et al*., 2004).

No presente trabalho de dissertação, foi utilizado um modelo onde o LPS de *P. gingivalis* foi administrado localmente na pata de ratos; neste modelo, a indução dos receptores B_1 cininérgicos foi avaliada *in vivo* e *ex vivo*. Ademais, foram investigados alguns dos mecanismos envolvidos no processo de indução do receptor B_1 após a aplicação de LPS de *P. gingivalis*.

OBJETIVOS

2. OBJETIVOS

2.1. Objetivo Geral

O objetivo do presente trabalho foi caracterizar o efeito do LPS de *P. gingivalis*, principal agente etiológico da doença periodontal, sobre a modulação dos receptores B_1 para as cininas na pata de ratos.

2.2 Objetivos Específicos

2.2.1. Verificar o padrão da resposta inflamatória induzida pela des-Arg^9-BK, um agonista seletivo dos receptores B_1, em patas de ratos previamente tratados com LPS de *P. gingivalis*;

2.2.2. Investigar o efeito do tratamento local com LPS de *P. gingivalis* sobre os níveis de RNAm para os receptores B_1 através do uso da técnica de PCR em tempo real (Real-time PCR);

2.2.3. Determinar farmacologicamente alguns dos possíveis mecanismos responsáveis pela indução dos receptores B_1 após o tratamento com LPS de *P. gingivalis*;

2.2.4. Determinar o efeito do tratamento local com LPS de *P. gingivalis* sobre os níveis de TNFα e sobre a migração de neutrófilos, na pata de ratos;

2.2.5. Avaliar o possível efeito modulatório do mediador lipídico pró-resolução resolvina E1 sobre a indução dos receptores B_1 após o tratamento com LPS de *P. gingivalis*.

ARTIGO CIENTÍFICO

3. ARTIGO CIENTÍFICO

Os resultados do presente trabalho foram submetidos ao The Journal of Immunology:

Resolvin E1 downregulates the effects of *P. gingivalis* LPS, a TLR2 activator, on the expression of kinin B_1 receptors

Fator de impacto (ISI Web of Knowledge): 6,068

Resolvin E1 downregulates the effects of *P. gingivalis* LPS, a TLR2 activator, on the expression of kinin B_1 receptors

Running title: TLR2 and *in vivo* B_1 receptor upregulation

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Keywords: Bacterial, Cytokines, Neutrophils, Inflammation, Rodent

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Abstract

It has been demonstrated that kinin B_1 receptors are highly upregulated under several stressful stimuli, such as infection. In spite of that, there is no evidence indicating whether *Porphyromonas gingivalis* LPS (Pg-LPS), a preferential TLR2 activator, might lead to B_1 receptor upregulation. In this study, we demonstrate that Pg-LPS injection into the rat paw resulted in a marked functional upregulation of B_1 receptors (as measured by an increase of B_1 receptor-induced edema formation), which was preceded by a rapid raise in B_1 receptor mRNA expression. The local administration of Pg-LPS also resulted in a prominent production of the proinflammatory cytokine TNF-α, followed by an increase of neutrophil influx; both events were observed at time periods prior to B_1 receptor induction. The functional and molecular Pg-LPS-elicited B_1 receptor upregulation was significantly reduced by the glucocorticoid dexamethasone, and to a lesser extent by the chimeric anti-TNFα antibody infliximab. Of high relevance, we show for the first time that a single administration of the pro-resolution lipid mediator Resolvin E1 was able to markedly down-regulate Pg-LPS-driven B_1 receptor expression, probably by inhibiting TNFα production and neutrophil migration to the inflammatory set. Collectively, the present findings clearly suggest that TLR2 activation by Pg-LPS is able to induce the upregulation of B_1 receptors, through mechanisms involving TNFα release and neutrophil influx, which are largely sensitive to Resolvin E1. It is tempting to suggest that kinin B_1 receptors might well represent a pivotal pathway for the inflammatory responses evoked by *P. gingivalis* and its virulence factors.

Introduction

Kinins are a group of biologically active peptides involved in several physiological and pathological conditions, such as vasodilatation, increased vascular permeability and cellular migration (1-3). The actions of kinins are mediated by the activation of two different G-protein coupled receptors, named B_1 and B_2 (1, 4, 5). B_2 receptors are generally expressed in a constitutive manner, mediating most of the physiological actions evoked by kinins, and exhibiting higher affinity for bradykinin (BK) and kallidin (1, 2). In contrast, B_1 receptors show high affinity for the active metabolites des-Arg^9-BK and des-Arg^{10}-KD. They are usually absent under normal conditions, but can be upregulated after tissue injury and during inflammatory and infectious diseases (1-3, 6).

Periodontal disease is a chronic infection that causes gingival inflammation and destruction of supporting structure of the teeth that leads to bone resorption and tooth loss (7-10). Specific groups of bacteria have been associated with periodontal destruction; among them, *Porphyromonas gingivalis* is a gram-negative strain that harbors well-known virulence factors with pathogenic potential. The host immune response to bacterial products, such as LPS, is a key to the establishment and progression of periodontal tissue destruction (9). Toll-Like Receptors (TLR) are pattern recognition receptors that distinguish microbial structures, which generate a cytokine-based responses involved in the induction of adaptative immunity. LPS from Gram-negative enterobacteria commonly signals through TLR4; on the other hand, *P. gingivalis* LPS (Pg-LPS) seems to signal mainly via TLR2 (11-13). TLR2 activation by Pg-LPS triggers

the downstream stimulation of a myriad of second messengers and transcription factors, leading to the release of pro-inflammatory cytokines, such as TNFα and IL-1β (14-16).

Few studies have demonstrated a possible connection between periodontal disease and kinins production, in a process involving B_2 receptor activation (17-19). In spite of that, there is no available evidence showing how TLR2 activation by Pg-LPS might lead to kinin B_1 receptor modulation. Therefore, this study was aimed at investigating whether Pg-LPS might induce functional and molecular upregulation of the kinin B_1 receptors. Furthermore, we evaluate for the first time, how the pro-resolving lipid mediator, Resolvin E1, can modulate kinin B_1 receptor modulation.

Materials and methods

Drugs and chemical reagents

The following drugs and reagents were used: LPS from *Porphyromonas gingivalis* (InvivoGen, San Diego, CA, USA); dexamethasone, EDTA, hexadecyltrimethyl ammonium bromide, tetramethylbenzidine, PMSF, benzamethonium chloride, aprotinin A (Sigma Chemical Co., St. Louis, MO, USA); Infliximab (Remicade®, Centocor, Horsham, PA, USA); Resolvin E1 (Cayman Chemical, Michigan, USA); des-Arg^9-BK (Bachem Bioscience, King of Prussia, PA); $NaPO_4$, hydrogen peroxide, NaCl, and Tween 20 (all from Merck, Haar, Germany). Most drugs were prepared and stocked in saline solution (NaCl 0.9 %) or phosphate buffered saline (PBS), except Resolvin E1 that was provided in absolute ethanol. The final concentration of ethanol never exceeded 0.1 % and did not display any effect *per se*.

Animals

Non-fasted male Wistar rats (6 to 8 per group, 140-180 g) obtained from Central Biotery of Universidade Federal de Pelotas (UFPEL, Brazil) were used in this study. The animals were housed in groups of five and maintained in a temperature (22 ± 2 ºC) and humidity-controlled room (60 - 80 %) with a 12/12 h light/dark cycle (lights on at 7:00AM), and food and water were available *ad libitum*. Rats were adapted at the laboratory for a period of 1 h prior to experimental procedures. Tests were performed between 8:00AM and 6:00PM. Each animal was used only once, and was immediately euthanized at the end of the experimental period by isoflurane inhalation. The reported experiments were conducted in accordance with current guidelines for the care of

laboratory animals and ethical guidelines for investigations of experiments in conscious animals (20) and were pre-approved by the Institutional Animals Ethics Comitee (CEUA-PUCRS).

B_1 receptor-mediated-rat paw edema

This series of experiments was accomplished according to the method described by Passos et al. (1), with minor modifications. Briefly, the animals received a 0.1 ml intradermal (i.d.) injection in one hind paw (right paw) of PBS (composition, 137 mmol/litre NaCl, 2.7 mmol/litre KCl, and 10 mmol/litre phosphate buffer) containing the selective kinin B_1 receptor agonist des-Arg^9-bradykinin (des-Arg^9-BK; 100 nmol/paw). The contralateral paw (left paw) received 0.1 ml of PBS and was used as the control. Edema was measured with a plethysmometer (Ugo Basile, Comerio, Italy), at several time points (10, 20, 30, 60, and 120 min) after injection of des-Arg^9-BK. The edema is expressed in millilitres (ml) as the difference between the right and left paws.

In most experiments, animals were locally pre-treated with LPS from *Porphyromonas gingivalis* (Pg-LPS; 3 μg/paw diluted in PBS; 1 to 72 h beforehand) at the same site of des-Arg^9-BK injection. Control animals received the same volume of PBS solution (0.1 ml). The dose of Pg-LPS was selected on the basis of pilot experiments and did not evoke any significant alteration of paw volume *per se*. In all experiments, the i.d. injections were performed under slight anesthesia with isoflurane (1 ml/ml).

Mechanisms responsible for functional B_1 upregulation in rats pre-treated with Pg-LPS

In order to determine some of the possible mechanisms underlying the upregulation of des-Arg^9-BK-induced paw edema following Pg-LPS local administration, separate groups of animals were systemically pre-treated with the anti-inflammatory steroid dexamethasone (0.5 mg/kg, s.c., 2 h before Pg-LPS administration), the chimeric monoclonal anti-TNFα antibody infliximab (1 mg/kg, s.c., 15 min before Pg-LPS), or the pro-resolution lipid mediator Resolvin E1 (RvE1, 300 ng/rat, i.p. 30 min before Pg-LPS). Control animals received the vehicle at the same schedules of treatment. The doses of inhibitors were selected in accordance with previous studies (21-23). In these experimental sets, des-Arg^9-BK (100 nmol/paw)-caused edema was evaluated 24 h after Pg-LPS (3 μg/paw) treatment as described before.

Expression of B_1 receptor mRNA in the rat paw

The expression of B_1 receptor mRNA was measured by Real-Time PCR, following the methodology described by Batista et al. (24). Rats were treated with Pg-LPS (3 μg/paw) and were euthanized at different intervals of time (1 to 5 h). PBS-treated paws were used as control. After euthanasia, the subcutaneous tissue of the paws was removed in RNAse free conditions and transferred to tubes containing RNA stabilization reagent (RNA later, Ambion). Immediately thereafter, the tissues were processed according to the protocol of a RNA purification commercial kit (RNeasy, Qiagen, Valencia, CA, USA). Tissues were initially frozen in liquid nitrogen and ground with mortar and pestle. RNA samples were then homogenized, and total RNA was isolated. All RNA samples were subjected to on-column DNAse I (Sigma-Aldrich) treatment to

remove trace amounts of genomic DNA. RNA concentrations and purity were determined spectrofotometrically at 260 and 260/280 nm, respectively. Samples presenting 260/280 ratios of 1.8 or higher were included in the analysis. RNA quality was assessed through formaldehyde denaturing 1.2 % agarose gels stained with SybrGold (Molecular Probes, Eugene, Oregon, USA) to check for the presence of clear 18s and 28s bands, and no smearing. For cDNA synthesis, 200 ng of total RNA was primed with random hexamers and reverse transcribed using an AML-V Reverse Transcriptase (TaqMan Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). Quantification of B_1 receptor mRNA was carried out through fluorescence-based Real-Time PCR. To this end, approximately 100 ng of cDNA were amplified in duplicates using TaqMan-based chemistry with specific primers and FAM-labeled probes for rat kinin B_1 receptor (Assays-on-Demand, Applied Biosystems, Foster City, CA, USA), and β-actin as an endogenous control for normalization (Endogenous Controls, Applied Biosystems, Foster City, CA, USA). The reaction plate was run in duplicate for every condition. Amplifications were carried out in a thermalcycler (ABI 7500, Applied Biosystems, Foster City, CA, USA) for 50 cycles; the fluorescence was collected at each amplification cycle and the data analysed using the $2^{-\Delta\Delta Ct}$ method for expression relative quantification as reported (Pfaffl, 2001). Before indicating this method, validation of the assays and efficiency of amplification of rat β-actin and kinin B_1 receptors were calculated through a 10-fold serial dilution of Pg-LPS-treated rat paw cDNA (not shown) as previously described (Livak and Schmittgen, 2001); the slope values of log input amounts plotted against ΔCt (Mean $Ct_{receptor}$ – Mean $Ct_{\beta Actin}$) for both, target gene and endogenous control, were found to be within acceptable values, making it suitable the use of the $2^{-\Delta\Delta Ct}$

method. Expression of the target genes was calibrated against conditions found in naive animal.

In a separated series of experiments, different groups of animals were pretreated with dexamethasone, infliximab, or Resolvin E1, at the same schemes of administration as described above. The control group was treated with the vehicle solution. Following the appropriate intervals of time for each drug, rats received an i.d. injection of Pg-LPS (3 μg/paw) and they were euthanized at 3 h. The procedures for Real-Time PCR were carried out as reported above.

Measurement of TNFα levels in the rat paw

TNFα production in the rat paw was measured as described by Passos et al. (21). The animals were locally treated with Pg-LPS (3 μg/paw; 1 to 3 h prior to euthanasia), and had the subcutaneous tissue of the right hindpaw removed and placed on a PBS solution containing 0.05 % Tween 20, 0.1 mM PMSF, 0.1 mM benzamethonium chloride, 10 mM EDTA, and 20 KI units of aprotinin A. PBS-treated paws were used as control. Tissues were homogenized and centrifuged at 5000 x g for 10 min, and the supernatant was collected and stored at -80 °C for further analysis. The levels of TNFα were evaluated using a standard sandwich Elisa protocol (R&D Systems DuoSet Kit).

Separate experimental groups were pretreated with dexamethasone, Resolvin E1, or vehicle solution, at the same doses and intervals of time, as described beforehand. Following 2 h of dexamethasone administration or 15 min of treatment with Resolvin E1, the animals received an i.d. injection of Pg-LPS (3 μg/paw), and they were euthanized 1

h later. The tissue processing and Elisa experiments were performed as described formerly.

Neutrophil myeloperoxidade (MPO) assay

Neutrophil recruitment to the rat paw was measured by means of tissue MPO activity, determined according to Souza et al (25). Animals received an i.d. injection of Pg-LPS (3 μg/paw) in the right paw and were euthanized at different intervals of time (1 to 36 h). PBS-treated paws were used as control. The subcutaneous tissue of the paws was removed, homogenized at 5 % (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 5000 g for 20 min at 4°C. The pellet was resuspended in 0.5 % hexadecyltrimethyl ammonium bromide buffer (pH 5.4) and the samples were frozen. Upon thawing, the samples were re-centrifuged and 25 μl of the supernatant were used for MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM NaPO4, and 0.3 mM hydrogen peroxide. The absorbance was measured at 595 nm, and the results are expressed as OD per milligram (mg) of tissue.

To determine some of the mechanisms responsible for the increased MPO activity following Pg-LPS i.d. administration, other groups of rats received dexamethasone, infliximab, Resolvin E1, or vehicle, at the same doses and time periods indicated beforehand. The biochemical assay for determining MPO activity was the same described in this section, and the tissues were collected at 3 h.

Statistical analysis

Most results are presented as the mean ± standard error of the mean (s.e.m.) of 5 to 8 animals per group. For the Real-Time PCR experiments, the results are given as the mean ± s.e.m of 3 independent experiments. Statistical comparison of the data was performed by one-way analysis of variance (one-way ANOVA) followed by Boferroni's test, or unpaired Student's *t*-test when appropriated. *P*-values smaller than 0.05 ($P < 0.05$) were considered as indicative of significance. Total inhibitions of the edematogenic responses are given as the difference (in percentage) between the areas under the time-response curve (AUC) of the drug-treated group in relation to the corresponding control group. The area under the curve was also utilized for demonstrating the time-related effects of Pg-LPS treatment on des-Arg^9-BK-induced edema formation.

Results

Modulation of kinin B_1 receptors after Pg-LPS treatment

Our data showed that i.d. injection of the selective kinin receptor agonist, des-Arg^9-BK (100 nmol/paw) in control animals produced a small increase in rat paw volume (0.24 ± 0.008 ml). In contrast, i.d administration of des-Arg^9-BK in animals locally pretreated with the TLR2 activator Pg-LPS (3 μg/paw; 3 to 72 h) induced a marked rat paw edema formation (Fig. 1A). The analysis of the AUC revealed that Pg-LPS effects on B_1 receptor-mediated edema present a time-related profile (Figure 1B). Accordingly, des-Arg^9-BK-evoked edema reached its peak between 6 and 36 h after Pg-LPS administration, decreasing following 72 h of Pg-LPS treatment. Therefore, the 24 h time-point (57 ± 9 % of increase) was chosen for the subsequent studies of functional B_1 receptor upregulation.

In another set of experiments, to evaluate some of the mechanisms implicated in kinin B_1 receptor regulation, we pre-treated animals with the anti-inflammatory steroid dexamethasone (0.5 mg/kg, s.c.) 2 h before Pg-LPS injection. The edema induced by des-Arg^9-BK (100 nmol/paw) in rats pretreated with Pg-LPS (3 μg/paw, 24 h previously) was significantly inhibited by the systemic administration of dexamethasone (Figure 2A). The percentage of inhibition observed for dexamethasone treatment was 39 ± 9 %, as calculated on the basis of the AUC (Figure 2B).

To determine the possible involvement of TNFα in Pg-LPS-induced B_1 receptor upregulation, we have employed the anti-TNFα chimeric monoclonal antibody infliximab (1 mg/kg, s.c.), dosed 15 min before Pg-LPS. Data revealed that des-Arg^9-BK (100 nmol/paw)-evoked edema following Pg-LPS treatment (3 μg/paw, 24 h) was

partially, but significantly inhibited by systemic pretreatment with infliximab (Figure 2C), with an inhibition percentage of 24 ± 5 %, as estimated on the basis of the AUC (Figure 2D).

Finally, we have evaluated to what extent the pro-resolution lipid mediator Resolvin E1 might prevent Pg-LPS-induced functional B_1 receptor upregulation. For this purpose, animals received a single i.p. injection of Resolvin E1 (300 ng/animal), 30 min before Pg-LPS treatment (3 μg/paw), and the edema was induced 24 h later by the B_1 receptor agonist des-Arg^9-BK. Noteworthy, this strategy was able to markedly decrease the edema formation elicited by des-Arg^9-BK (100 nmol/paw) in Pg-LPS (3 μg/paw)-injected rats (Figure 1E), with an inhibition percentage of 43 ± 7 %, as calculated using the AUC (Figure 2F).

B_1 Receptor mRNA expression

The changes in kinin B_1 receptor mRNA expression following Pg-LPS local treatment are presented in Figure 3A. Bar graphs represent variations relative to control animals; therefore, they reflect fold-changes relative to basal levels of B_1 receptor mRNA. The intraplantar injection of Pg-LPS (3 μg/paw) produced a marked and time-related increase of B_1 receptor mRNA expression in the rat subcutaneous paw tissue, which was evident as early as at 1 h following LPS administration (5-fold increase), and reached its peak at 3 h afterhand (17-fold increase), decreasing after 5 h. Therefore, in assessing Pg-LPS B1 receptor expression in response to different anti-inflammatory compounds we adopted the 3-hour time point as the cut-off. Pretreating the animals with the glucocorticoid dexamethasone was able to reduce the expression of B_1 mRNA by 85

± 4 %, infliximab reduced the expression B_1 mRNA by 61 ± 23% and, of high interest, B_1 receptor mRNA expression was virtually abolished by the pro-resolution lipid mediator Resolvin E1 (96 ± 3 %) (Figure 3B).

TNF-α levels

TNFα is a pro-inflammatory cytokine with multiple biological actions, which is upregulated by infectious stimuli (26). The relevance of TNFα production for the upregulation of kinin B_1 receptors in the rat paw was further assessed by ELISA analysis, at different intervals of time (1 to 5 h) after Pg-LPS local treatment. The results depicted in Figure 4A indicate that Pg-LPS administration induced a significant increase in TNF-α levels in the subcutaneous paw tissue, which reached maximal effect at 1 h (about 8-fold). In contrast, undetectable or very low levels of TNFα were found in control animals. Noteworthy, the administration of dexamethasone (0.5 mg/kg, s.c., 2 h prior to Pg-LPS), and Resolvin E1 (300 ng/animal, 30 min before Pg-LPS) significantly reduced the augmentation of TNFα levels in response to Pg-LPS, as shown in Figure 4B. The percentages of inhibition of TNFα production were 54 ± 7.5 % and 96 ± 4 %, respectively.

Relevance of neutrophil influx

The migration of neutrophils to the rat paw in response to Pg-LPS (3 μg/paw) treatment was evaluated indirectly by means of MPO activity assay. As shown in Figure 5A, Pg-LPS injection (1 to 36 h) was capable of inducing a time-related increase in MPO levels, reaching the maximal values between 3 and 12 h, with an approximated 2-fold

augmentation, in comparison to control group. The increase in MPO activity at 3 h was significantly reduced by the pretreatment with dexamethasone (0.5 mg/kg, s.c., 2 h prior to Pg-LPS) and Resolvin E1 (300 ng/animal, 30 min before Pg-LPS) The inhibition indexes for these treatments were 45 ± 6 % and 55 ± 3 %, respectively. On the other hand, this inflammatory parameter was not significantly modified by treatment with infliximab (1 mg/kg, s.c., 15 min before Pg-LPS) (Figure 5B).

Discussion

Kinin B_1 receptors are atypical G protein-coupled receptors, which are generally absent under physiological conditions, being highly and quickly upregulated following inflammatory and infectious stimuli (3). The induction of this receptor has been associated with the production of inflammatory cytokines, neutrophil migration and activation of several intracellular signaling pathways (5, 21, 27). There is compelling evidence showing that kinin B_1 receptors can be upregulated after treatment with infectious stimuli*,* either *in vivo* or *in vitro* (3, 28). To our best knowledge, there are only some few reports linking periodontal disease and kinin B_2 receptor activation (18, 19, 29, 30). In spite of that, there is no evidence showing whether Pg-LPS, a TLR2 activator, might lead to the upregulation of B_1 receptors. The results of the present study provide, for the first time, convincing evidence showing that kinin B_1 receptor can be up-regulated after *in vivo* administration of the TLR2 activator Pg-LPS, by mechanisms sensitive to the pro-resolving lipid mediator Resolvin E1.

A previous publication from our group demonstrated that local treatment with LPS from *E. coli* resulted in a rapid and prolonged functional upregulation of B_1 receptors in the rat paw, as assessed by the increase in paw edema in response to the selective B_1 agonist des-Arg^9-BK (21). In this publication, the authors demonstrated that edema caused by des-Arg^9-BK peaked at 12 h, and then decreased gradually between 24 and 36 h after *E. coli* LPS administration. Of interest, the results obtained in the present study show that Pg-LPS local administration can induce a marked increase in the rat paw edema caused by des-Arg^9-BK, an effect that peaked between 12 h and 36 h, remaining significant for up to 72 h. Literature data have suggested that LPS from *E. coli*

preferentially activates TLR4 receptors, whereas Pg-LPS displays a greater affinity for TLR2 receptors (14-16). Furthermore, some recent publications have pointed to the activation of differential signaling pathways following the stimulation of TLR2 and TLR4 receptors (11-13). Concerning the B_1 receptor modulation, it seems that both Pg-LPS and LPS from *E. coli* are able to produce the functional upregulation of B_1 receptors, although slight temporal and intensity differences are observed for these responses. Accordingly, the maximal increase of des-Arg9-BK-induced paw edema was about 206 % for *E. coli* LPS at 12 h, whereas the enhancement of this response was 54 %, for Pg-LPS at 24 h.

Dexamethasone is a glucocorticoid that displays several anti-inflammatory actions via either genomic or non-genomic means (31). In this regard, there is compelling evidence indicating that glucocorticoids might block TLR signaling pathways via multiple mechanisms of action (32). In relation to B_1 receptors, several studies has demonstrated that dexamethasone is able to block their upregulation induced by infectious stimuli, such as *E. coli* LPS (3, 21). Data provided in this study are in accordance with the literature and point out that dexamethasone markedly reduces des-Arg^9-BK-induced edematogenic response after Pg-LPS treatment. Therefore, it is possible to imply that *in vivo* upregulation of B_1 receptors in our model, induced by local administration of Pg-LPS is related to *de novo* protein synthesis. This idea was further confirmed by data showing that time-dependent upregulation of B_1 receptor-mediated paw edema after Pg-LPS local treatment, was preceded by a rapid and transient increase of kinin B_1 receptor mRNA expression in the rat paw (between 1 and 5 h), as assessed by

real-time PCR experiments. It is worth mentioning that Pg-LPS-induced increase of B_1 receptor mRNA was markedly prevented by pretreating animals with dexamethasone.

Our results demonstrate that chimeric anti-TNFα antibody infliximab produced a partial, but significant inhibition of edema formation induced by des-Arg^9-BK in Pg-LPS-pretreated rats. This evidence allows us to suggest that B_1 receptor functional upregulation by Pg-LPS is a process dependent, at least in part, on the release of the inflammatory cytokine TNFα. The relevance of TNFα for functional upregulation of B_1 receptors has been also demonstrated elsewhere (21, 33, 34). Furthermore, extending our *in vivo* data, we were also able to demonstrate that increase of kinin B_1 receptor mRNA expression following Pg-LPS treatment was sensitive to the systemic treatment with the anti-TNFα antibody infliximab. Relevantly, it has been recently shown that TLR2 activation by Pg-LPS leads to TNFα upregulation, by the stimulation of transduction pathways distinct from that of *E. coli* LPS; whereas TLR2/JNK is the main pathway for Pg-LPS, the induction by *E. coli* LPS is likely mediated by TLR4/NF-κB/ p38MAPK pathways (35). Further studies are still required to clarify what intracellular signaling pathways are involved in kinin B_1 receptor upregulation by Pg-LPS.

Resolvin E1 is an endogenous lipid mediator biosynthesized from the precursor essential omega-3 polyunsaturated fatty acid, eicosapentaenoic acid (EPA). It has been demonstrated that Resolvin E1 orchestrates the resolution of inflammatory processes, by promoting tissue return to homeostasis (36). Accordingly, Resolvin E1 prevents neutrophil infiltration, and also stimulates the phagocytic activity of macrophages, increasing the exit of inflammatory cells from the inflamed site and antimicrobial defense mechanisms (36, 37). Previous results on a rabbit model of periodontitis demonstrated an

important role for Resolvin E1 in the inhibition of alveolar bone resorption (38). Interestingly, a recent *in vitro* study demonstrated that Resolvin E1 is able to limit osteoclast growth and bone resorption by interfering with osteoclast differentiation (39). In this paper, we describe further mechanisms of Resolvin E1 actions in the inflammatory responses evoked by periodontal pathogens, on the subcellular level. Noteworthy, data provided in the present study show, for the first time, the ability of the pro-resolving mediator Resolvin E1 in reducing des-Arg^9-BK-evoked paw edema, in animals pretreated with Pg-LPS. In addition, the administration of a single dose of Resolvin E1 almost abolished the increase of B_1 receptor mRNA expression elicited by Pg-LPS. On the basis of our results, it is tempting to suggest that anti-inflammatory effects of Resolvin E1 in experimental models of periodontitis are, at least partially, related to the inhibition of B_1 receptor upregulation.

Another interesting result of our study indicate that Pg-LPS injection into the rat paw results in a marked and time-related increase of TNFα production, which was maximal at 1 h after Pg-LPS administration. Of interest, this temporal profile was very similar to that observed after *E. coli* LPS treatment in the rat paw tissue, where TNFα levels were maximal following 1 h, in a process sensitive to dexamethasone treatment (Passos et al., 2004). In our study, enhance of TNF α levels in response to Pg-LPS injection were significantly diminished by dexamethasone and Resolvin E1. Also important, it has been demonstrated that Resolvin E1 was able to reduce leukocyte infiltration, and to prevent TNFα gene expression in an experimental model of colitis in mice (40). In this context, our findings suggest that part of the inhibitory effects of

Resolvin E1 on B_1 receptor upregulation rely on the reduction of TNFα production in the rat paw.

Determination of MPO activity is a widely accepted hallmark of neutrophil migration to the sites of inflammation (25, 26). Several pieces of evidence have indicated a correlation between neutrophil migration and the upregulation of kinin B_1 receptors (41, 42). Hence, previous data demonstrate that injection *E. coli* LPS into the rat paw induced a time-related increase in MPO levels, which peaked at 12 h and lasted for up to 36 h (21). The results provided herein show that administration of Pg-LPS induced a marked and earlier increase of MPO activity, which was found significant at 3 h after Pg-LPS injection, and remained increased for up to 12 h. The temporal profile for the augmentation of MPO activity by Pg-LPS was consistent with the upregulation of B_1 receptor-mediated edema formation, what suggests that neutrophils might provide signals for the modulation of kinin B_1 receptors. Interestingly, the elevation of MPO activity induced by Pg-LPS was significantly reduced by pretreatment with dexamethasone, although infliximab failed to significantly affect this parameter. On the basis of this set of experimental data, it is possible to surmise that TNFα increase and neutrophil influx are independent factors that contribute to the upregulation of B_1 receptors by Pg-LPS. In our study, we also demonstrated that Resolvin E1 markedly reduced the increase of MPO activity in response to Pg-LPS injection. These results are consistent with the previous evidence showing that Resolvin E1 is able to prevent neutrophil infiltration (36, 38, 43). It is alluring to propose that inhibitory effects of Resolvin E1 on B_1 receptor modulation are possibly related to the inhibition of neutrophil migration.

Altogether, the present data suggest the following sequence of events leading to B_1 receptor upregulation after the local administration of the TLR2 activator Pg-LPS *in vivo*: there is a rapid increase of TNFα generation, followed by increased neutrophil migration, conveying to the upregulation of B_1 receptors. Of interest, the pro-resolution mediator Resolvin E1 is likely able to dowregulate B_1 receptor expression, by reducing both TNFα release and neutrophil influx (Figure 6). These findings shed new lights on the mechanisms underlying kinin B_1 receptor modulation, and indicate that this receptor subtype might represent a relevant pathway for the inflammatory responses evoked by *P. gingivalis* and its virulence factors.

Acknowledgements

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Figures:

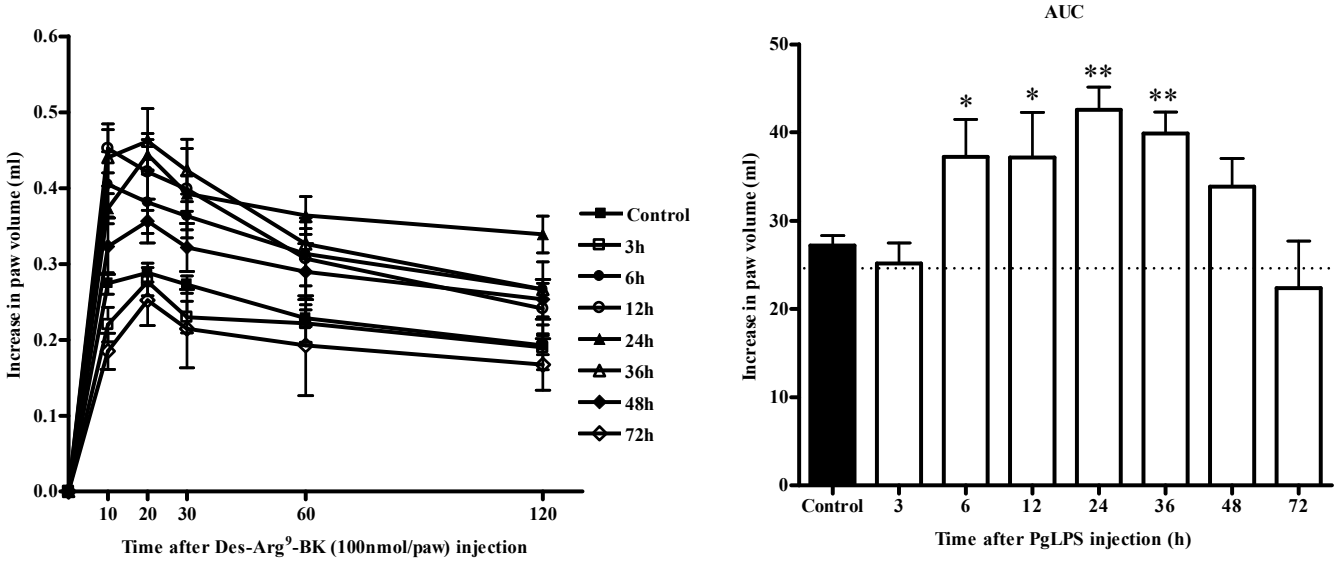

Figure 1. Time-dependent effects of Pg-LPS (3 μg/paw) treatment on B_1 receptor upregulation in the rat paw. (A) Increase in the rat paw volume in response to des-Arg^9-BK (100 nmol/paw) injection in Pg-LPS and PBS-treated animals. Values represent the difference (ml) between PBS- and Pg-LPS-treated paws. (B) AUC for the time-related effects of Pg-LPS on B_1 receptor-mediated paw edema. Each point represents the mean ± SEM of five animals. * $p< 0,05$; ** $p< 0,01$.

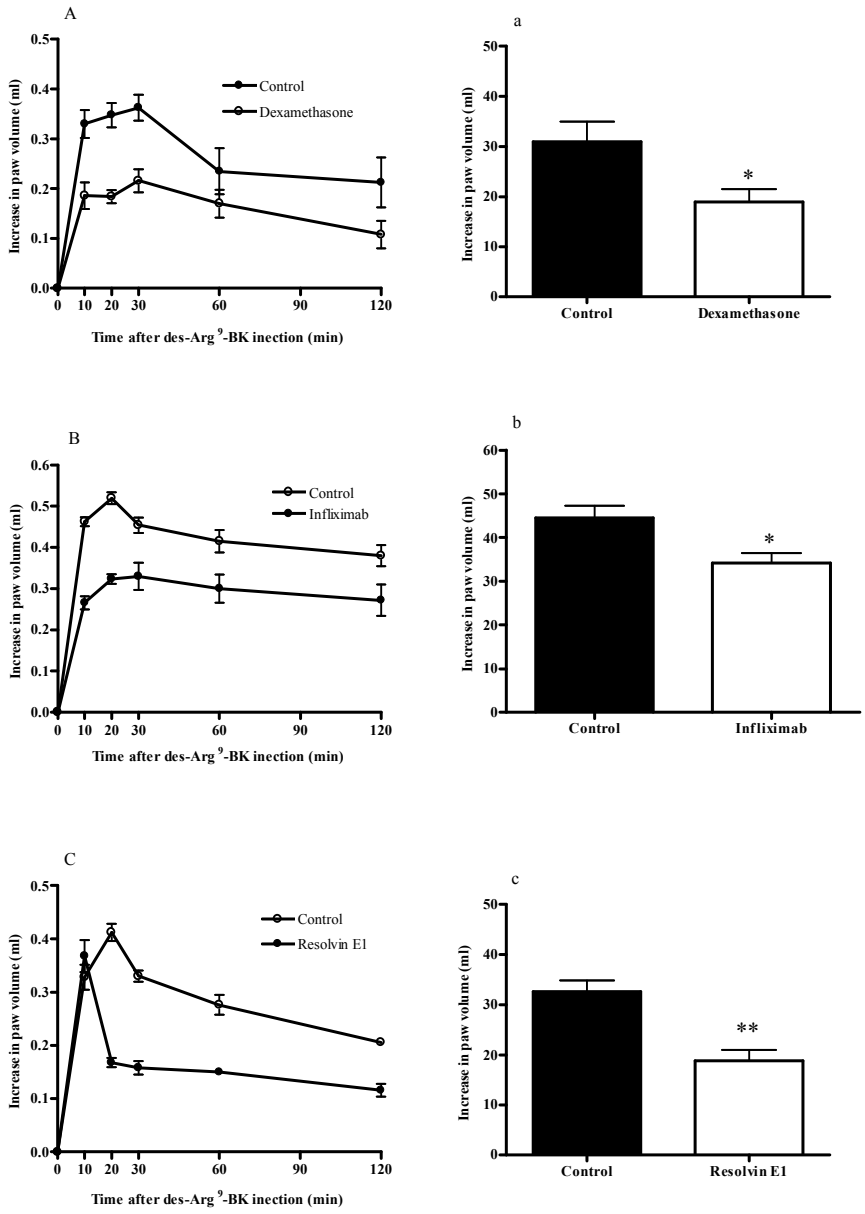

Figure 2. **Effects of some pharmacological inhibitors on des-Arg^9-BK (100 nmol/paw)-induced paw edema in rats that have been treated with Pg-LPS (3 μg/paw, 24 h).** (A) Effect of treatment with dexamethasone (0.5 mg/kg, s.c., 2 h). (B) Effect of treatment with infliximab (1 mg/kg, s.c., 15 min). (C) Effect of treatment with Resolvin E1 (300 ng/rat, i.p., 30 min). (a), (b) and (c) represent the AUC for each inhibitor. Each point represents the mean ± SEM of five animals. * p< 0,05; ** p< 0,01.

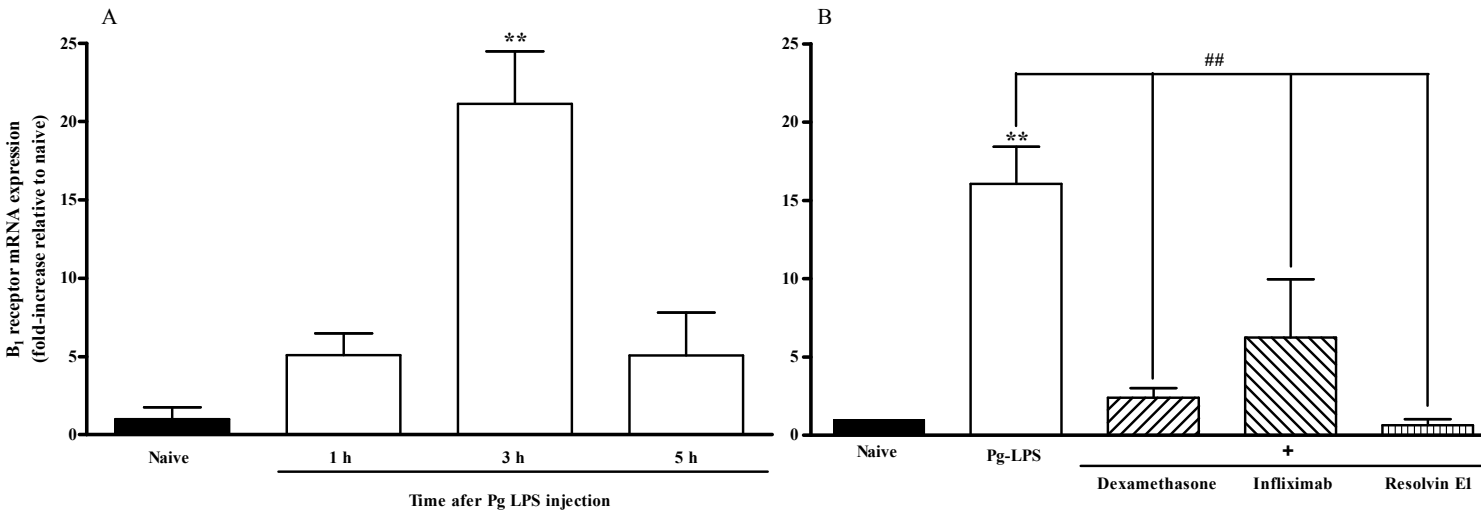

Figure 3. Effects of Pg-LPS on kinin B_1 receptor mRNA expression. (Panel A) Time-related effect of Pg-LPS (3 μg/paw) injection on B_1 receptor mRNA expression in the rat paw. (Panel B) Effects of treatment with: dexamethasone (0.5 mg/kg, s.c., 2 h), infliximab (1 mg/kg, s.c., 15 min) or Resolvin E1 (300 ng/rat, I.p., 30 min) on B_1 receptor mRNA expression in rats pretreated with Pg-LPS (3 μg/paw, 3 h). Each column represents the mean of three independent experiments and the vertical lines the s.e.m. Significantly different from control (**) or LPS-injected (##) paws, $p < 0.01$.

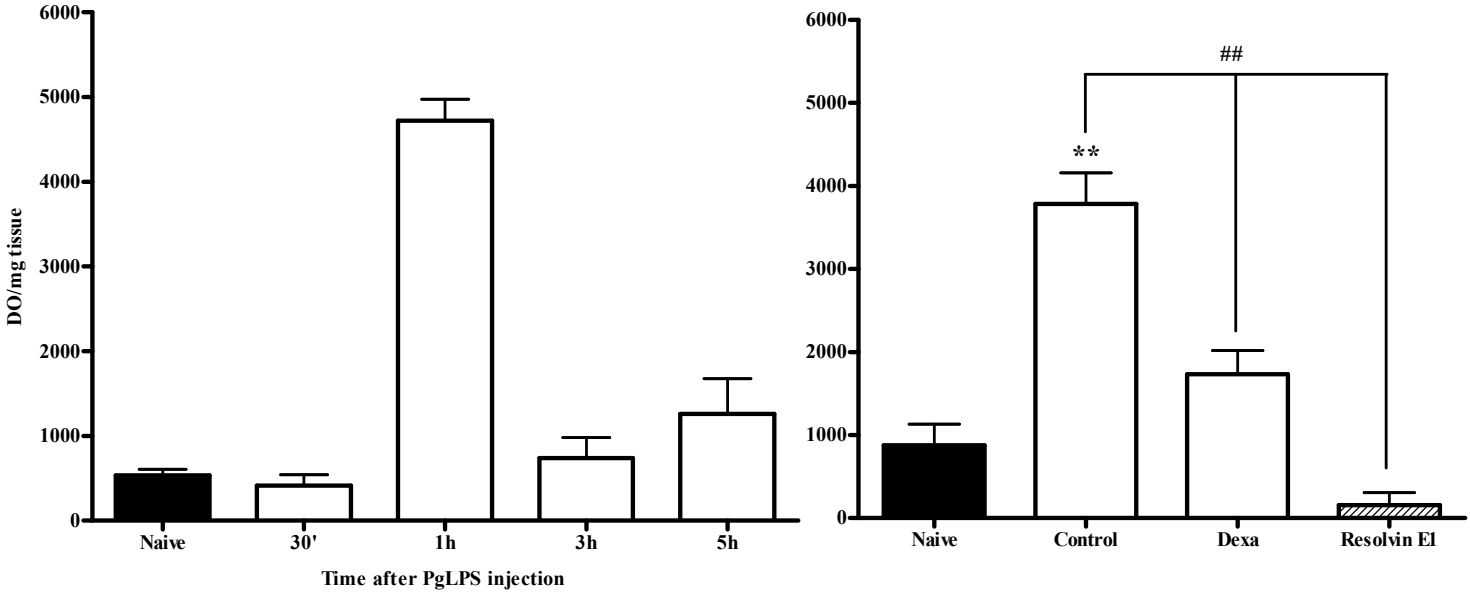

Figure 4. **Effects of Pg-LPS on TNFα production.** (A) Time-related effect of Pg-LPS (3 μg/paw) injection on TNFα levels in the rat paw. (B) Effects of treatment with dexamethasone (0.5 mg/kg, s.c., 2 h) or Resolvin E1 (300 ng/rat, I.p., 30 min) on TNFα levels in animals pretreated with PgLPS (3 μg/paw,1 h). Each point represents the mean ± SEM of five animals. Significantly different from control (**) or LPS-injected paws (##), $p < 0.01$.

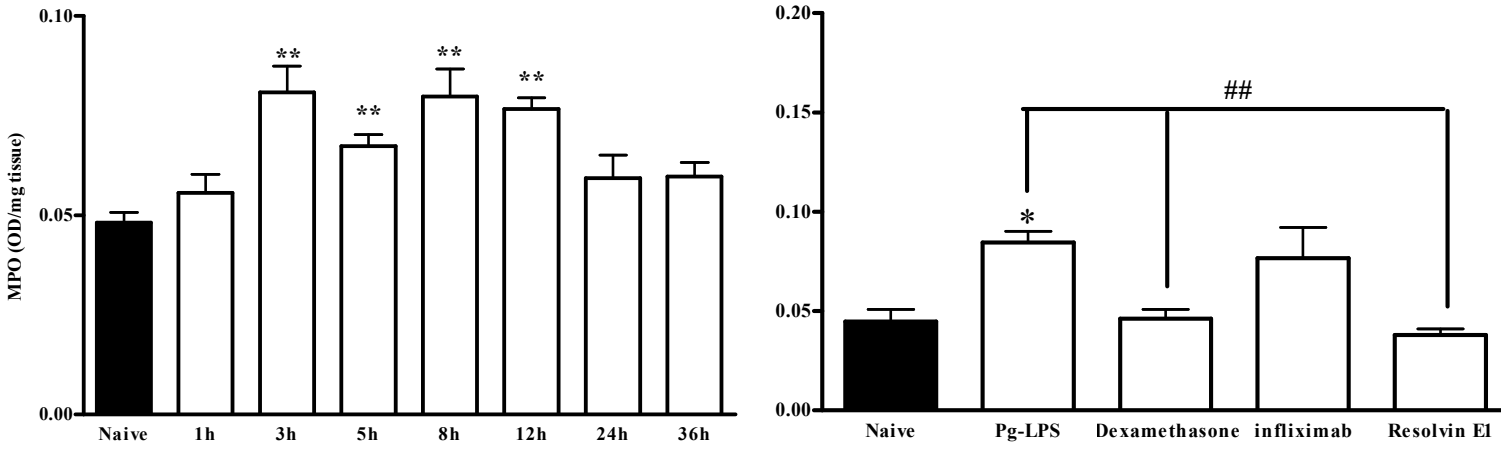

Figure 5. Effects of Pg-LPS on MPO activity in the rat paw tissue. (A) Time-related effect of Pg-LPS (3 μg/paw) on MPO levels in the rat paw; LPS was injected 1 to 36 h before. (B) Effects of treatment with: dexamethasone (0.5 mg/kg, s.c., 2 h), infliximab (1 mg/kg, s.c., 15 min) or Resolvin E1 (300 ng/rat, i.p., 30 min) on neutrophil migration in animals pretreated with Pg-LPS (3 μg/paw, 3 h). Each point represents the mean ± SEM of five animals. Significantly different from control (**) or LPS-injected paws (##), $p < 0.01$.

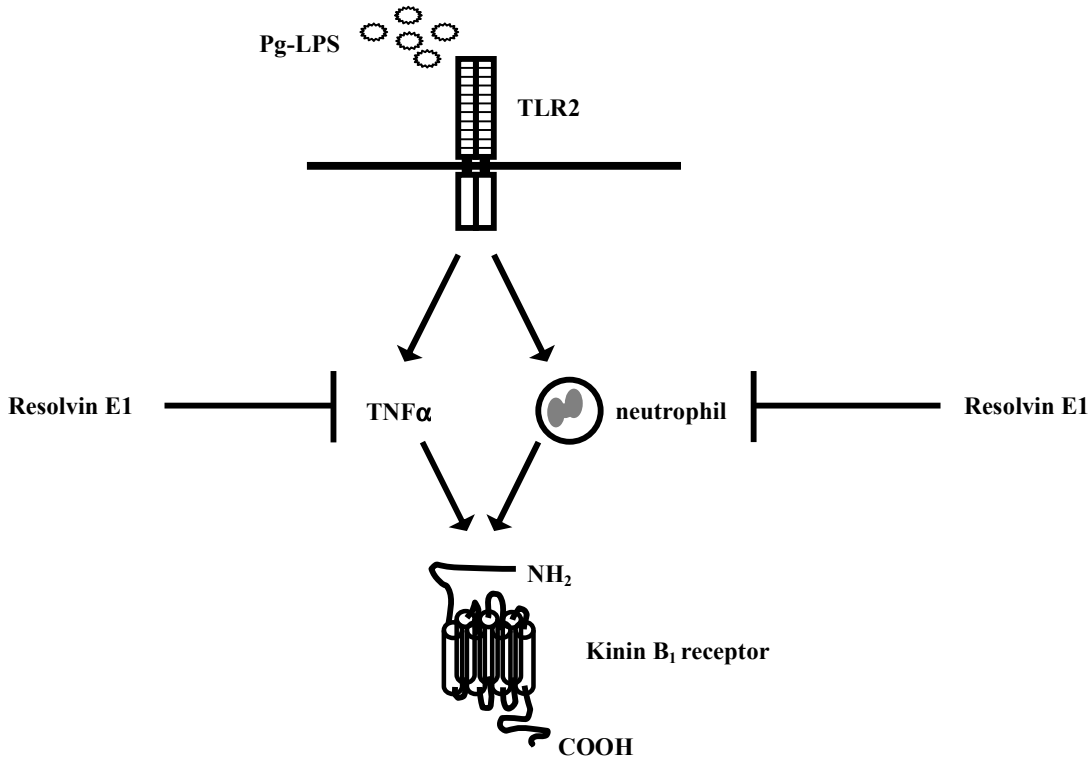

Figure 6. Schematic representation of possible main mechanisms underlying kinin B_1 receptor upregulation in Pg-LPS-treated rats.

CONSIDERAÇÕES FINAIS

4. CONSIDERAÇÕES FINAIS

Os receptores B_1 para as cininas apresentam um padrão de expressão complexo, diferente da maioria dos receptores acoplados à proteína G. Estão normalmente ausentes na maior parte dos órgãos e tecidos, com exceção do sistema nervoso central, podendo ser rapidamente induzidos e modulados em diversos tipos celulares, após trauma tecidual ou alterações patológicas (Calixto et al., 2004). Seu caráter induzido apresenta semelhanças ao padrão de expressão de receptores ligados a tirosina-quinase. Evidências sugerem que a expressão desses receptores é um processo altamente regulado, compreendendo tanto mecanismos transcricionais quanto pós-transcricionais (Campos et al., 2006). A melhor definição dessas vias de sinalização intracelular é de grande importância para a descoberta de novos alvos terapêuticos e da patofisiologia de várias doenças.

A indução dos receptores B_1 tem sido associada à produção de mediadores inflamatórios, recrutamento de células inflamatórias e ativação de diversas vias de sinalização intracelular. Ademais, tem sido demonstrado que a regulação dos receptores B_1 envolve um processo sensível a inibidores de síntese protéica, sugerindo mecanismos relacionados à síntese *de novo* destes receptores (Calixto et al., 2004).

Como mencionado anteriormente, processos infecciosos constituem estímulos clássicos para a indução dos receptores B_1 (Marceau *et al*., 1998; Calixto *et al*., 2000; 2001; 2004). Um estudo conduzido por DeBlois e Horlick (2001), em macacos, indicou que o agonista seletivo dos receptores B_1, a des-Arg^9-BK, é capaz de induzir edema de pele, apenas em animais pré-tratados com LPS de *E. coli*. Além disso, foi demonstrado que o LPS de *E. coli* produz um aumento marcante do extravasamento plasmático

induzido pela des-Arg^9-BK no duodeno, na traquéia e no íleo de ratos (Wille *et al*., 2001). Mais recentemente, Passos e colaboradores (2004) mostraram a ocorrência de indução dos receptores B_1 após a administração local de LPS de *E. coli* na pata de ratos, em um processo dependente da ativação do fator de transcrição NF-κB, da migração de neutrófilos, da liberação das citocinas IL-1β e TNFα e do mediador lipídico PAF (fator de ativação plaquetária). Outros trabalhos recentes conduzidos *in vitro* mostraram que a incubação contínua com LPS de *E. coli* em preparações de traquéia de camundongo (Bachar *et al*., 2004), veia porta de rato (Medeiros *et al*., 2004) ou íris de porco (El Sayah *et al*., 2006) é capaz de induzir um aumento tempo-dependente das respostas contráteis mediadas pelos receptores B_1 para as cininas.

As ações do LPS de *E. coli* estão associadas com a ativação de receptores TLR4 e com a subseqüente estimulação do fator nuclear NF-κB e de várias proteínas quinases, além da produção de citocinas pró-inflamatórias, sendo todos esses sinais relacionados com a indução dos receptores B_1 (Dobrovolskaia e Vogel, 2002; Akira *et al.,* 2003). Entretanto, estudos recentes têm demonstrado que o LPS de *P. gingivalis* desencadeia a resposta imune inata, preferencialmente através da estimulação do receptor TLR2 (Burns *et al*., 2006, Kikkert *et al*., 2007, Hajishengallis *et al*., 2008). Cabe ressaltar, que até o momento, não havia estudos correlacionando a ativação de receptores TLR2 pelo LPS de *P. gingivalis*, com a indução dos receptores B_1 para as cininas.

O presente trabalho procurou avaliar a seqüência de eventos que leva ao aumento da expressão dos receptores B_1 após a administração *in vivo* de LPS de *P. gingivalis* através de estudos funcionais e de biologia molecular. A partir dos resultados obtidos nesse trabalho, pode-se sugerir que o tratamento local com LPS de *P. gingivalis* promove

uma liberação da citocina pró-inflamatória TNFα, seguida do influxo de neutrófilos para o sitio inflamatório, resultando no aumento da expressão do receptor B_1. De grande interesse, foi demonstrado pela primeira vez, que o mediador lipídico pró-resolução da inflamação, Resolvina E1, parece ser capaz de modular a expressão dos receptores B_1, provavelmente por interferir com a liberação de TNFα e com a migração de neutrófilos.

Algumas publicações recentes demonstraram a ocorrência da ativação de vias de sinalização distintas após a estimulação de receptores TLR2 e TLR4 (Burns et al., 2006; Zhou et al., 2007; Hajishengallis et al., 2008). Em relação aos receptores B_1, os resultados do presente estudo sugerem que tanto o LPS de *E. coli*, quanto o LPS de *P. gingivalis*, são capazes de induzir o aumento das respostas mediadas pelos receptores B_1 cininérgicos, embora algumas diferenças temporais e de intensidade tenham sido observadas.

Quando um determinado evento inflamatório é desencadeado por bactérias, vírus ou trauma tecidual, o principal objetivo da resposta imune do hospedeiro é retornar rapidamente à homeostasia através da remoção das células leucocitárias do sítio inflamatório. A resolução completa do processo inflamatório ocorre após a remoção dos leucócitos do sítio inflamatório por macrófagos fagocíticos, ocasionando a total eliminação do patógeno invasor. Quando o processo de resolução não ocorre efetivamente, a inflamação torna-se crônica. Em condições como a periodontite, o controle do influxo de neutrófilos pode evitar que um processo agudo de gengivite evolua para uma inflamação crônica como a periodontite (Kornman, 2008).

A resolução da inflamação não é o mesmo que a antiinflamação. A resolução é um processo ativo que necessita de ativação de moléculas pró-resolução, a fim de

neutralizar e eliminar os leucócitos inflamatórios. Já os agentes antiinflamatórios agem bloqueando ou inibindo a produção de mediadores inflamatórios, como as prostaglandinas e leucotrienos, reduzindo os sinais clássicos da inflamação. Entretanto, as drogas antiinflamatórias podem interferir com o processo de resolução, pois prolongam a inflamação tecidual e o tempo de resolução da lesão. No caso da doença periodontal, quando a resposta imune do hospedeiro à placa bacteriana não é eficiente, ocorre destruição tecidual e reabsorção do osso alveolar (Van Dyke, 2008; Williams, 2008).

A Resolvina E1 é um mediador lipídico endógeno derivado do ácido graxo polinsaturado omega-3, ácido ecosapentanóico (EPA), que parece orquestrar o processo de resolução da inflamação promovendo o retorno do tecido à homeostasia. Tem sido demonstrado que a Resolvina E1 é capaz de prevenir o influxo de neutrófilos no sítio inflamatório além de estimular a ação fagocítica dos macrófagos aumentando a saída das células inflamatórias bem como os mecanismos de defesa contra microorganismos (Serhan et al., 2008; Van Dyke, 2008).

Trabalhos anteriores demonstraram a capacidade da Resolvina E1 em interferir com a diferenciação de osteoclastos, em reduzir o influxo de neutrófilos para o sítio inflamatório, bem como em diminuir a produção de mediadores da inflamação, tais como o TNFα (Arita *et al*., 2005; Hasturk *et al*., 2007; Herrera *et al*., 2008). Entretanto, até o presente não havia dados da literatura mostrando como um mediador pró-resolução seria capaz de prevenir a indução dos receptores B_1 para as cininas. Neste sentido, os resultados do presente estudo mostram, pela primeira vez, que o LPS de *P. gingivalis* é capaz de modular a indução funcional e molecular dos receptores B_1, através de mecanismos sensíveis ao mediador da resolução, Resolvina E1. Com base nos dados aqui

apresentados é possível inferir que: (i) a indução dos receptores B_1 pode representar um fator determinante nas alterações inflamatórias causadas por patógenos associados com a doença periodontal; (ii) parte dos efeitos antiinflamatórios exibidos pela Resolvina E1 em modelos animais de doença periodontal podem estar relacionados ao bloqueio dos receptores B1; (iii) antagonistas seletivos dos receptores B_1 poderiam representar alternativas interessantes para o controle da doença periodontal.

5. RESULTADOS ADICIONAIS

Nesta seção, são descritos alguns resultados adicionais que não fizeram parte do manuscrito submetido à publicação:

5.1. MATERIAIS E MÉTODOS:

5.1.1. Determinação do edema de pata induzido pelo agonista seletivo dos receptores B_1, des-Arg^9-BK, após o tratamento com *P. gingivalis* inativada por calor (Heat Killed *Porphyromonas gingivalis*-HKPg)

Os animais receberam uma injeção intraplantar de 0,1 ml de PBS contendo HKPg (10^8células/pata), na pata direita. Após diferentes intervalos de tempo (3 a 144 h), os animais receberam uma injeção intraplantar do agonista B_1, des-Arg^9-BK, na mesma pata e solução salina na pata esquerda. Para a determinação do edema de pata, foi usada a metodologia descrita por Passos *et al* (2004). O edema foi medido em pletismômetro (Ugo Basile), em vários intervalos de tempo (10 – 120 min) e o resultado foi expresso em ml, como a diferença de volume entre as patas direita e esquerda.

5.1.2. Influência do pré-tratamento com inibidores

Com o objetivo de avaliar alguns dos mecanismos implicados na resposta edematogênica induzida pela des-Arg^9-BK, na pata de ratos pré-tratados com HKPg (10^8células/ pata, 48 h), os animais foram tratados com dexametasona (0,5 mg/kg, s.c., 2 h antes da injeção de HKPg) ou com o anticorpo monoclonal anti-TNFα, infliximab (1 mg/kg, s.c., 15 min antes da injeção de HKPg). Animais controle foram tratados apenas com HKPg. O edema foi determinado como descrito acima e as respostas obtidas foram

analisadas comparando-se o edema produzido pela des-Arg^{9}-BK após tratamento com HKPg na presença ou ausência de inibidores farmacológicos.

5.2. RESULTADOS

5.2.1. Caracterização da resposta edematogênica causada pelo agonista seletivo para os receptores B_1 para as cininas em animais pré-tratados com HKPg.

Como demonstrado anteriormente (Passos *et al*., 2004), a injeção intraplantar do agonista seletivo para os receptores B_1, a des-Arg^9-BK (100 nmol/pata) produziu apenas uma discreta alteração na pata de animais controle (27,17 ± 1,15 %; área sob a curva). Por outro lado, a injeção de des-Arg^9-BK em animais que foram previamente tratados com HKPg (10^8 células/pata) resultou em um aumento marcante no volume das patas, sendo a resposta máxima observada 48 h após a injeção da HKPg (43,42 ± 2,91%)

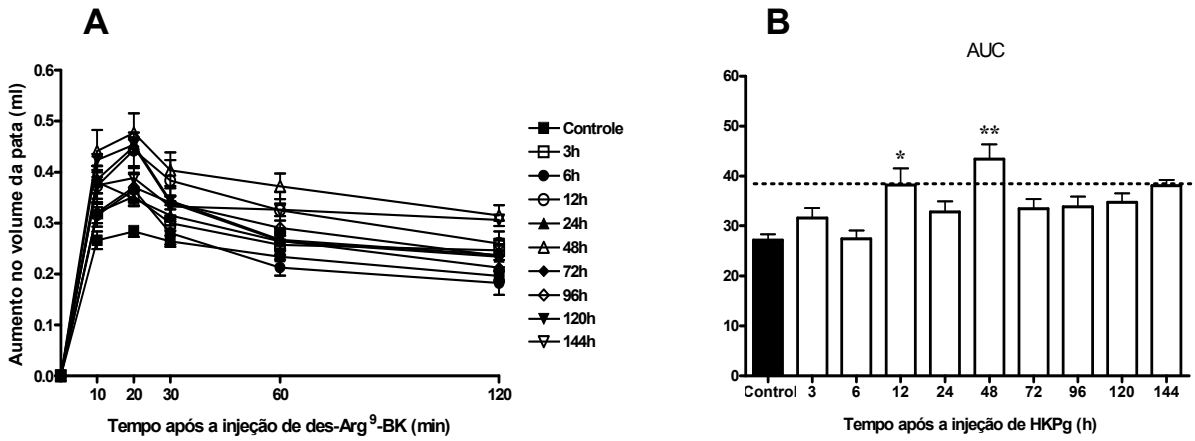

Figura 1: Efeito do pré-tratamento com HKPg na resposta edematogênica mediada pelos receptores B_1 na pata de rato. (A) Edema de pata induzido pelo agonista seletivo para os receptores B_1, a des-Arg^9-BK (100 nmol/pata) em animais controle ou pré-tratados com HKPg (10^8 células/pata), determinado em diferentes intervalos de tempo (1-144h). (B) Área sob a curva para o efeito tempo-dependente da HKPg sobre os receptores

B_1. Cada ponto representa a média ± e.p.m. de 5 animais. $^{*}p<0,05$, $^{**}p<0,01$ em relação ao grupo controle (PBS).

5.2.2. Análise dos mecanismos envolvidos no edema de pata causado pela des-Arg^9-BK em animais pré-tratados com HKPg

O aumento na resposta edematogênica induzido pela des-Arg^9-BK (100 nmol/pata) em animais pré-tratados com HKPg foi inibido de forma significativa pela administração prévia do glicocorticóide dexametasona ou do anticorpo monoclonal anti-TNFα infliximab. As percentagens de inibição observadas foram: 39 ± 3%, 47 ± 2 %. respectivamente (área sob a curva).

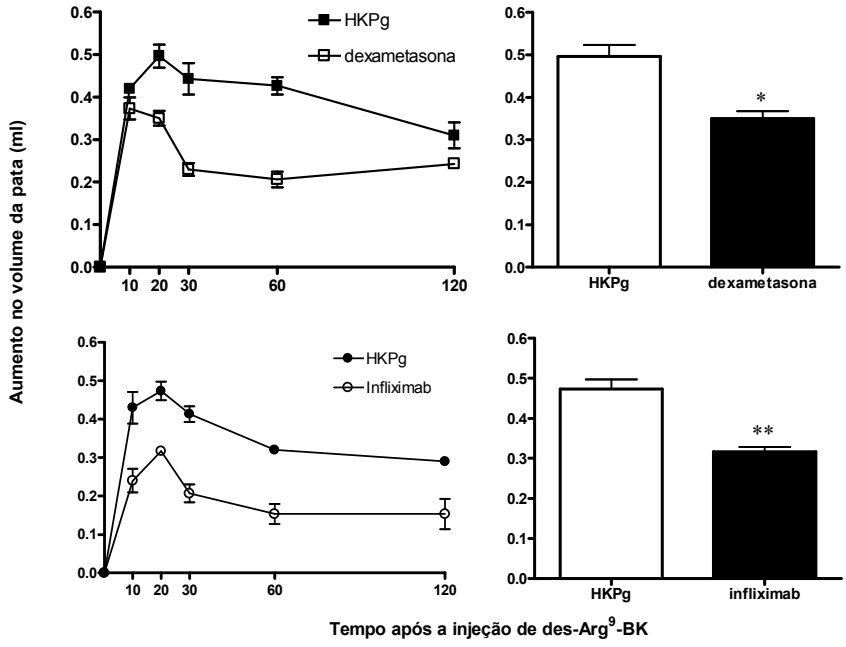

Figure 2. Efeito de inibidores farmacológicos sobre o edema de pata induzido pela des-Arg^9-BK (100 nmol/paw) em animais pré-tratados com HKPg (10^8 células/pata) (A) Efeito do tratamento com dexametasona (0.5 mg/kg, s.c., 2 h). (B) Efeito do tratamento com infliximab (1 mg/kg, s.c., 15 min). (a) e (b) representam a area sob a curva para cada inibidor. Cada ponto representa a média ± e.p.m de 5 animais. * $p< 0,05$; ** $p< 0,01$.

PARTICIPAÇÃO EM OUTROS TRABALHOS E PROJETOS

6. PARTICIPAÇÃO EM OUTROS TRABALHOS E PROJETOS

1. TRABALHOS PUBLICADOS:

PEREIRA, P.J., **DORNELLES, F.N**., SANTOS, D.S., CALIXTO, J.B., MORRONE, F.B., CAMPOS, M.M. Nociceptive and inflammatory responses induced by formalin in the orofacial region of rats: effect of anti-TNFα strategies. International Immunopharmacology. 2009; 9:80-85.

2. OUTROS PROJETOS:

Caracterização do papel dos receptores B_1 e B_2 das cininas nas alterações comportamentais induzidas por LPS de *E. coli* em camundongos

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8. ANEXOS

PEREIRA, P.J., **DORNELLES, F.N**., SANTOS, D.S., CALIXTO, J.B., MORRONE, F.B., CAMPOS, M.M. Nociceptive and inflammatory responses induced by formalin in the orofacial region of rats: effect of anti-TNFα strategies. International Immunopharmacology. 2009; 9:80-85.

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In Vivo Up-Regulation of Kinin B_1 Receptors after Treatment with *Porphyromonas gingivalis* Lipopolysaccharide in Rat Paw

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ABSTRACT

It has been demonstrated that kinin B_1 receptors are highly up-regulated under several stressful stimuli, such as infection. However, there is no evidence indicating whether *Porphyromonas gingivalis* lipopolysaccharide (Pg-LPS) might lead to B_1 receptor up-regulation. In this study, we demonstrate that Pg-LPS injection into the rat paw resulted in a marked functional up-regulation of B_1 receptors (as measured by an increase of B_1 receptor-induced edema), which was preceded by a rapid rise in B_1 receptor mRNA expression. The local administration of Pg-LPS also resulted in a prominent production of the proinflammatory cytokine tumor necrosis factor α (TNF-α), followed by an increase of neutrophil influx; both events were observed at periods before B_1 receptor induction. The functional and molecular Pg-LPS-elicited B_1 receptor up-regulation was significantly reduced by the glucocorticoid dexamethasone (0.5 mg/kg s.c.), and to a lesser extent by the chimeric anti-TNF-α antibody infliximab (1 mg/kg s.c.). Of high relevance, we show for the first time that a single administration of the proresolution lipid mediator (5*S*,12*R*,18*R*)-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid (resolvin E1; 300 ng/rat i.p.) was able to markedly down-regulate Pg-LPS-driven B_1 receptor expression, probably by inhibiting TNF-α production and neutrophil migration. Collectively, the present findings clearly suggest that Pg-LPS is able to induce the up-regulation of B_1 receptors through mechanisms involving TNF-α release and neutrophil influx, which are largely sensitive to resolvin E1. It is tempting to suggest that kinin B_1 receptors might well represent a pivotal pathway for the inflammatory responses evoked by *P. gingivalis* and its virulence factors.

Kinins are a group of biologically active peptides involved in several physiological and pathological conditions, such as vasodilatation, increased vascular permeability, and cellular migration (Calixto et al., 2004). The actions of kinins are mediated by the activation of two different G-protein-coupled receptors, named B_1 and B_2 (Marceau et al., 1998; Calixto et al., 2004). In general, B_2 receptors are expressed in a constitutive manner, mediating most of the physiological actions evoked by kinins, and exhibiting higher affinity for bradykinin (BK) and kallidin (Calixto et al., 2004). In contrast, B_1 receptors show high affinity for the active metabolites des-Arg^9-BK and des-Arg^{10}-kallidin. They are usually absent under normal conditions, but can be up-regulated after tissue injury and during inflammatory and infectious diseases (Marceau et al., 1998; Calixto et al., 2004).

Periodontal disease is a chronic infection that causes gingival inflammation and destruction of the supporting structure of the teeth, leading to bone resorption and tooth loss (Nishida et al., 2001; Carayol et al., 2006; Ohno et al., 2008). Specific groups of bacteria have been associated with periodontal destruction; among them, *Porphyromonas gingivalis* is a Gram-negative strain that harbors well known virulence factors with pathogenic potential. The host immune response

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ABBREVIATIONS: AUC, area under the time-response curve; BK, bradykinin; MPO, myeloperoxidase; LPS, lipopolysaccharide; Pg-LPS, *Porphyromonas gingivalis* LPS; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; ELISA, enzyme-linked immunosorbent assay; resolvin E1, RvE1, (5*S*,12*R*,18*R*)-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid.

to bacterial products, such as lipopolysaccharide (LPS), is a key to the establishment and progression of periodontal tissue destruction (Carayol et al., 2006). Toll-like receptors (TLRs) are pattern recognition receptors that distinguish microbial structures, which generate cytokine-based responses involved in the induction of adaptive immunity. LPS from Gram-negative enterobacteria commonly signals through TLR4; however, *P. gingivalis* LPS (Pg-LPS) seems to signal mainly via TLR2 (Burns et al., 2006; Zhou and Amar, 2007; Hajishengallis et al., 2008). TLR2 activation by Pg-LPS triggers the downstream stimulation of a myriad of second messengers and transcription factors, leading to the release of proinflammatory cytokines, such as TNF-α and interleukin-1β (Muthukuru et al., 2005; Kikkert et al., 2007). Few studies have demonstrated a possible connection between periodontal pathogens and kinin production in a process involving B_2 receptor activation (Imamura et al., 2004; Hu et al., 2006; Brechter et al., 2008); nevertheless, there is no available evidence showing how Pg-LPS might lead to kinin B_1 receptor modulation.

The rat paw edema is a very well characterized model for studying the in vivo up-regulation of B_1 receptors after several stimuli, including the local administration of LPS from *Escherichia coli* (Campos et al., 2006). The present study was aimed at investigating whether the local treatment with the periodontal pathogen Pg-LPS might induce functional and molecular up-regulation of the kinin B_1 receptors by use of the rat paw edema experimental paradigm.

Resolvin E1, recently identified as an omega-3 eicosapentaenoic acid derivative lipid mediator generated during the resolution phase of inflammation, has proved to be a potent inhibitor of neutrophil migration and cytokine production in vivo (Serhan et al., 2000, 2002). In our study, we have also evaluated how resolvin E1 can modulate kinin B_1 receptors up-regulation after Pg-LPS local administration.

Materials and Methods

Drugs and Chemical Reagents. The following drugs and reagents were used: LPS from *P. gingivalis* (InvivoGen, San Diego, CA); dexamethasone, EDTA, hexadecyltrimethyl ammonium bromide, tetramethylbenzidine, phenylmethylsulfonyl fluoride, benzamethonium chloride, aprotinin A (Sigma-Aldrich, St. Louis, MO); infliximab (Remicade, Centocor, Horsham, PA); resolvin E1 (Cayman Chemical, Ann Arbor, MI); des-Arg^9-BK (*H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH*; Bachem Bioscience, King of Prussia, PA); $NaPO_4$, hydrogen peroxide, NaCl, and Tween 20 (all from Merck, Haar, Germany). Most drugs were prepared and stocked in saline solution (NaCl 0.9%) or phosphate-buffered saline (PBS), except resolvin E1 that was provided in absolute ethanol. The final concentration of ethanol never exceeded 0.1% and did not display any effect per se.

Animals. In this study, nonfasted male Wistar rats (6–8 per group, 140–180 g) obtained from the Central Biotery of Universidade Federal de Pelotas (Brazil) were used. The animals were housed in groups of five and maintained in a temperature (22 ± 2°C)- and humidity-controlled room (60–80%) with a 12/12 h light/dark cycle (lights on at 7:00 AM), and food and water were available ad libitum. Rats were adapted at the laboratory for a period of 1 h before experimental procedures. Tests were performed between 8:00 AM and 6:00 PM. Each animal was used only once, and was immediately euthanized at the end of the experimental period by isoflurane inhalation. The reported experiments were conducted in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experiments in conscious animals (Zimmermann, 1983) and were preapproved by the Institutional Animals Ethics Committee (Comitê de Ètica para o Uso de Animais-Pontificia Universidade Católica do Rio Grande do Sul).

B_1 Receptor-Mediated Rat Paw Edema. This series of experiments was accomplished according to the method described by Passos et al. (2004), with minor modifications. In brief, the animals received a 0.1-ml intradermal (i.d.) injection in one hind paw (right paw) of PBS (composition: 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer) containing the selective kinin B_1 receptor agonist des-Arg^9-bradykinin (des-Arg^9-BK; 100 nmol/paw). The contralateral paw (left paw) received 0.1 ml of PBS and was used as the control. Edema was measured with a plethysmometer (Ugo Basile, Comerio, Italy), at several time points (10, 20, 30, 60, and 120 min) after injection of des-Arg^9-BK. The edema is expressed in milliliters as the difference between the right and left paws.

In most experiments, animals were locally pretreated with Pg-LPS (3 μg/paw diluted in PBS; 1–72 h beforehand) at the same site of des-Arg^9-BK injection. Control animals received the same volume of PBS solution (0.1 ml). The dose of Pg-LPS was selected on the basis of pilot experiments and did not evoke any significant alteration of paw volume per se. In all experiments, the intradermal injections were performed under slight anesthesia with isoflurane (1 ml/ml). Technical information on Pg-LPS used in the present study indicates that at low concentrations (<1 μg/ml), it induces TLR2 activation, and at higher concentrations (≥10 μg/ml), it induces marked TLR2, and weaker TLR4 stimulation (Darveau et al., 2004).

Mechanisms Responsible for Functional B_1 Up-Regulation in Rats Pretreated with Pg-LPS. To determine some of the possible mechanisms underlying the up-regulation of des-Arg^9-BK-induced paw edema after Pg-LPS local administration, separate groups of animals were pretreated systemically with the anti-inflammatory steroid dexamethasone (0.5 mg/kg s.c., 2 h before Pg-LPS administration), the chimeric monoclonal anti-TNF-α antibody infliximab (1 mg/kg s.c., 15 min before Pg-LPS), or the proresolution lipid mediator resolvin E1 (RvE1, 300 ng/rat i.p., 30 min before Pg-LPS). Control animals received the vehicle at the same schedules of treatment. In these experimental sets, edema caused by des-Arg^9-BK (100 nmol/paw) was evaluated 24 h after Pg-LPS (3 μg/paw) treatment, as Pg-LPS-induced functional up-regulation of B_1 receptors peaked between 6 and 36 h.

The doses of inhibitors were selected based on previous studies and pilot experiments. These doses were demonstrated to be effective in different in vivo models of inflammation (Passos et al., 2004; Schwab et al., 2007; Seadi Pereira et al., 2009).

Expression of B_1 Receptor mRNA in the Rat Paw. The expression of B_1 receptor mRNA was measured by real-time PCR, following the methodology described by Batista et al. (2005). Rats were treated with Pg-LPS (3 μg/paw) and were euthanized at different intervals of time (1–5 h). PBS-treated paws were used as control. After euthanasia, the subcutaneous tissue of the paws was removed in RNase-free conditions and transferred to tubes containing RNA stabilization reagent (RNA later; Ambion, Austin, TX). Immediately thereafter, the tissues were processed according to the protocol of a RNA purification commercial kit (RNeasy; QIAGEN, Valencia, CA). Tissues were initially frozen in liquid nitrogen and ground with a mortar and pestle. RNA samples were then homogenized, and total RNA was isolated. All RNA samples were subjected to on-column DNase I (Sigma-Aldrich) treatment to remove trace amounts of genomic DNA. RNA concentrations and purity were determined spectrophotometrically at 260 and 260:280 nm, respectively. Samples presenting 260:280 ratios of 1.8 or higher were included in the analysis. RNA quality was assessed through formaldehyde denaturing 1.2% agarose gels stained with SybrGold (Molecular Probes, Eugene, OR) to check for the presence of clear 18S and 28S bands, and no smearing. For cDNA synthesis, 200 ng of total RNA was primed with random hexamers and reverse transcribed with use of an AML-V Reverse Transcriptase (TaqMan Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Quantification of B_1 re-

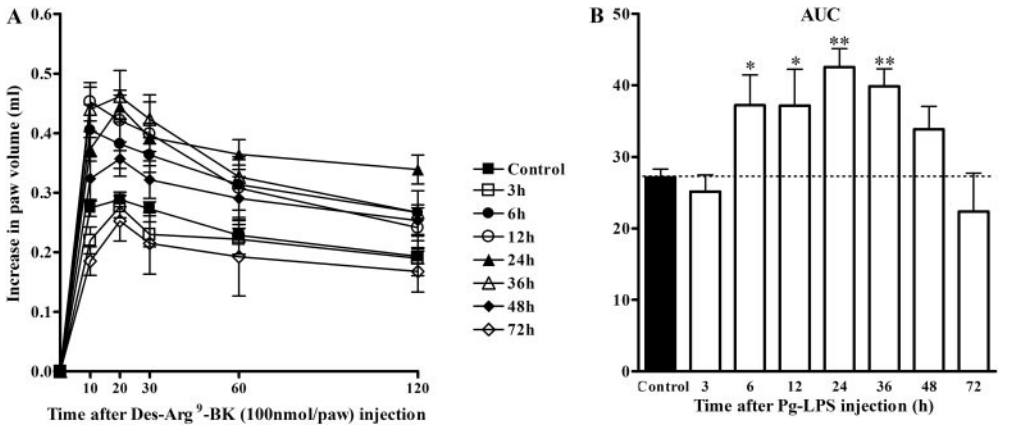

Fig. 1. Time-dependent effects of Pg-LPS (3 μg/paw) treatment on B_1 receptor up-regulation in the rat paw. A, increase in the rat paw volume in response to des-Arg^9-BK (100 nmol/paw) injection in Pg-LPS- and PBS-treated animals. Values represent the difference (milliliters) between PBS- and Pg-LPS-treated paws. B, AUC for the time-related effects of Pg-LPS on B_1 receptor-mediated paw edema. Each point represents the mean ± S.E.M. of five animals. *, $p < 0.05$; **, $p < 0.01$.

ceptor mRNA was performed through fluorescence-based real-time PCR. To this end, approximately 100 ng of cDNA were amplified in duplicates by use of TaqMan-based chemistry with specific primers and FAM-labeled probes for rat kinin B_1 receptor (Assays-on-Demand, Applied Biosystems), and β-actin as an endogenous control for normalization (Endogenous Controls, Applied Biosystems). The reaction plate was run in duplicate for every condition. Amplifications were performed in a thermal cycler (ABI 7500, Applied Biosystems) for 50 cycles; the fluorescence was collected at each amplification cycle and the data analyzed by the $2^{-\Delta\Delta Ct}$ method for expression relative quantification. Before indicating this method, validation of the assays and efficiency of amplification of rat β-actin and kinin B_1 receptors were calculated through a 10-fold serial dilution of Pg-LPS-treated rat paw cDNA (not shown); the slope values of log input amounts plotted against ΔCt (mean $Ct_{receptor}$ − mean $Ct_{\beta Actin}$) for both, target gene and endogenous control, were found to be within acceptable values, making it suitable for the use of the $2^{-\Delta\Delta Ct}$ method. Expression of the target genes was calibrated against conditions found in naive animals.

In a separate series of experiments, different groups of animals were pretreated with dexamethasone or resolvin E1, at the same schemes of administration as described above. The control group was treated with the vehicle solution. After the appropriate intervals of time for each drug, rats received an injection of Pg-LPS (3 μg/paw i.d.) and they were euthanized at 3 h. This time point was selected taking into consideration the maximal up-regulation of B_1 receptor mRNA after Pg-LPS administration. The procedures for real-time PCR were performed as reported above.

Measurement of TNF-α Levels in the Rat Paw. TNF-α production in the rat paw was measured as described by Passos et al. (2004). The animals were locally treated with Pg-LPS (3 μg/paw; 1–3 h before euthanasia), and had the subcutaneous tissue of the right hindpaw removed and placed on a PBS solution containing 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamethonium chloride, 10 mM EDTA, and 20 KI units of aprotinin A. PBS-treated paws were used as control. Tissues were homogenized and centrifuged at 5000*g* for 10 min, and the supernatant was collected and stored at −80°C for further analysis. The levels of TNF-α were evaluated via a standard sandwich ELISA protocol (DuoSet Kit; R&D Systems, Minneapolis, MN).

Separate experimental groups were pretreated with dexamethasone, infliximab, resolvin E1, or vehicle solution, at the same doses and intervals of time, as described earlier. After 2 h of dexamethasone administration or 15 min of treatment with resolvin E1, the animals received an injection of Pg-LPS (3 μg/paw i.d.), and they were euthanized 1 h later. This time point was selected considering the maximal increase of TNF-α levels, after local treatment with Pg-LPS. The tissue processing and ELISA experiments were performed as described previously.

Neutrophil Myeloperoxidase Assay. Neutrophil recruitment to the rat paw was measured by means of tissue myeloperoxidase (MPO) activity, determined according to Passos et al. (2004). Animals received an injection of Pg-LPS (3 μg/paw i.d.) in the right paw and were euthanized at different intervals of time (1–36 h). PBS-treated paws were used as control. The subcutaneous tissue of the paws was removed, homogenized at 5% (w/v) in EDTA/NaCl buffer, pH 4.7, and centrifuged at 5000*g* for 20 min at 4°C. The pellet was resuspended in 0.5% hexadecyltrimethyl ammonium bromide buffer, pH 5.4, and the samples were frozen. Upon thawing, the samples were recentrifuged and 25 μl of the supernatant were used for MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM $NaPO_4$, and 0.3 mM hydrogen peroxide. The absorbance was measured at 595 nm, and the results are expressed as optical density per milligram of tissue.

To determine some of the mechanisms responsible for the increased MPO activity after Pg-LPS intradermal administration, other groups of rats received dexamethasone, infliximab, resolvin E1, or vehicle, at the same doses and time periods indicated earlier. The biochemical assay for determining MPO activity was the same as described in this section. The tissues were collected at 3 h, because the increase of MPO activity peaked between 3 and 12 h after Pg-LPS administration.

Statistical Analysis. Most results are presented as the mean ± S.E.M. of five to eight animals per group. For the real-time PCR experiments, the results are given as the mean ± S.E.M. of three independent experiments. Statistical comparison of the data was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's test, or unpaired Student's *t* test when appropriate. *P* values smaller than 0.05 ($P < 0.05$) indicated significance. Total inhibitions of the edematogenic responses are given as the difference (in percentage) between the areas under the time-response curve (AUC) of the drug-treated group in relation to the corresponding control group. The area under the curve was also used for demonstrating the time-related effects of Pg-LPS treatment on des-Arg^9-BK-induced edema formation.

Results

Modulation of Kinin B_1 Receptors after Pg-LPS Treatment. Our data show that intradermal injection of the selective kinin receptor agonist des-Arg^9-BK (100 nmol/paw) in control animals produced a small increase in rat paw volume (0.24 ± 0.008 ml). In contrast, intradermal administration of des-Arg^9-BK in animals locally pretreated with Pg-LPS (3 μg/paw; 3–72 h) resulted in marked formation of rat paw edema (Fig. 1A). The analysis of the AUC revealed that Pg-LPS effects on B_1 receptor-mediated edema present a time-related profile (Fig. 1B). Accordingly, des-Arg^9-BK-evoked edema reached its peak between 6 and 36 h after Pg-LPS administration, decreasing after 72 h of Pg-LPS treatment. Therefore, the 24-h time point (57 ± 9% of in-

crease) was chosen for the subsequent studies of functional B_1 receptor up-regulation.

In another set of experiments, to evaluate some of the mechanisms implicated in kinin B_1 receptor regulation, we pretreated animals with the anti-inflammatory steroid dexamethasone (0.5 mg/kg s.c.) 2 h before Pg-LPS injection. The edema induced by des-Arg^9-BK (100 nmol/paw) in rats pretreated with Pg-LPS (3 μg/paw, 24 h previously) was significantly inhibited by the systemic administration of dexamethasone (Fig. 2A). The percentage of inhibition observed for dexamethasone treatment was 39 ± 9%, as calculated on the basis of the AUC. To determine the possible involvement of TNF-α in Pg-LPS-induced B_1 receptor up-regulation, we have used the anti-TNF-α chimeric monoclonal antibody infliximab (1 mg/kg s.c.), dosed 15 min before Pg-LPS. Data revealed that des-Arg^9-BK (100 nmol/paw)-evoked edema after Pg-LPS treatment (3 μg/paw, 24 h) was partially, but significantly inhibited by systemic pretreatment with infliximab (Fig. 2B), with an inhibition percentage of 24 ± 5%, as estimated based on the AUC. Finally, we have evaluated to what extent the proresolution lipid mediator resolvin E1 might prevent Pg-LPS-induced functional B_1 receptor up-regulation. For this purpose, animals received a single intraperitoneal injection of resolvin E1 (300 ng/animal), 30 min before Pg-LPS treatment (3 μg/paw), and the edema was induced 24 h later by the B_1 receptor agonist des-Arg^9-BK. This strategy was able to markedly decrease the edema formation elicited by des-Arg^9-BK (100 nmol/paw) in rats injected with Pg-LPS (3 μg/paw) (Fig. 2C), with an inhibition percentage of 43 ± 7%, as calculated by use of the AUC.

B_1 Receptor mRNA Expression. The changes in kinin B_1 receptor mRNA expression after Pg-LPS local treatment are presented in Fig. 3A. Bar graphs represent variations relative to control animals; therefore, they reflect fold changes relative to basal levels of B_1 receptor mRNA. The intraplantar injection of Pg-LPS (3 μg/paw) produced a marked and time-related increase of B_1 receptor mRNA expression in the rat subcutaneous paw tissue, which was evident as early as at 1 h after LPS administration (5-fold increase), and reached its peak at 3 h after (17-fold increase), decreasing after 5 h. Therefore, in assessing Pg-LPS B_1 receptor expression in response to different anti-inflammatory compounds we adopted the 3-h time point as the cutoff. Pretreatment of the animals with the glucocorticoid dexamethasone was able to reduce the expression of B_1 mRNA by 85 ± 4%; infliximab reduced the expression of B_1 mRNA by 61 ± 23%. Note that B_1 receptor mRNA expression was virtually abolished by the proresolution lipid mediator resolvin E1 (96 ± 3%) (Fig. 3B).

TNF-α Levels. TNF-α is a proinflammatory cytokine with multiple biological actions, which is up-regulated by infectious stimuli (Rocha et al., 2006). The relevance of TNF-α production for the up-regulation of kinin B_1 receptors in the rat paw was further assessed by ELISA analysis, at different intervals of time (1–5 h) after Pg-LPS local treatment. The results depicted in Fig. 4A indicate that Pg-LPS administration induced a significant increase in TNF-α levels in the subcutaneous paw tissue, which reached maximal effect at 1 h (approximately 8-fold). In contrast, undetectable or very low levels of TNF-α were found in control animals. The administration of dexamethasone (0.5 mg/kg s.c., 2 h before Pg-LPS), or resolvin E1 (300 ng/animal, 30 min before Pg-

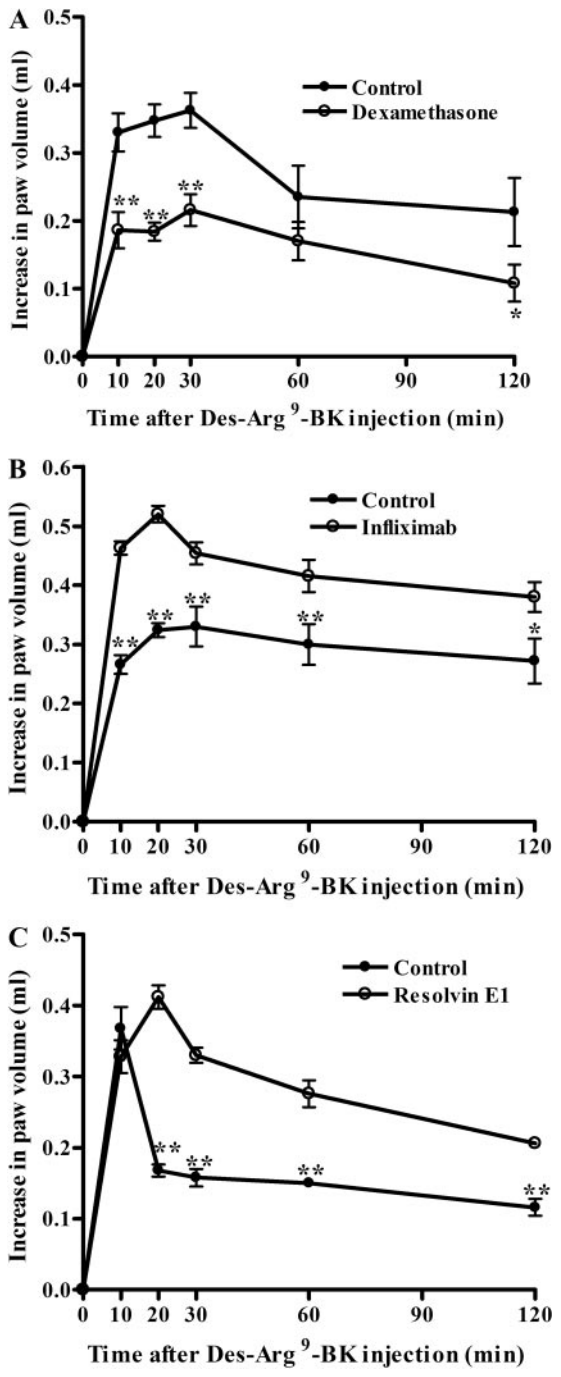

Fig. 2. Effects of some pharmacological inhibitors on des-Arg^9-BK (100 nmol/paw)-induced paw edema in rats that have been treated with Pg-LPS (3 μg/paw, 24 h). A, effect of treatment with dexamethasone (0.5 mg/kg s.c., 2 h). B, effect of treatment with infliximab (1 mg/kg s.c., 15 min). C, effect of treatment with resolvin E1 (300 ng/rat i.p., 30 min). Each point represents the mean ± S.E.M. of five animals. *, $p < 0.05$; **, $p < 0.01$.

LPS) significantly reduced the augmentation of TNF-α levels in response to Pg-LPS (as shown in Fig. 4B); the percentages of inhibition were 54 ± 7.5% and 96 ± 4%, respectively. However, infliximab failed to significantly affect TNF-α production (results not shown).

Relevance of Neutrophil Influx. The migration of neutrophils to the rat paw in response to Pg-LPS (3 μg/paw) treatment was evaluated indirectly by means of MPO activ-

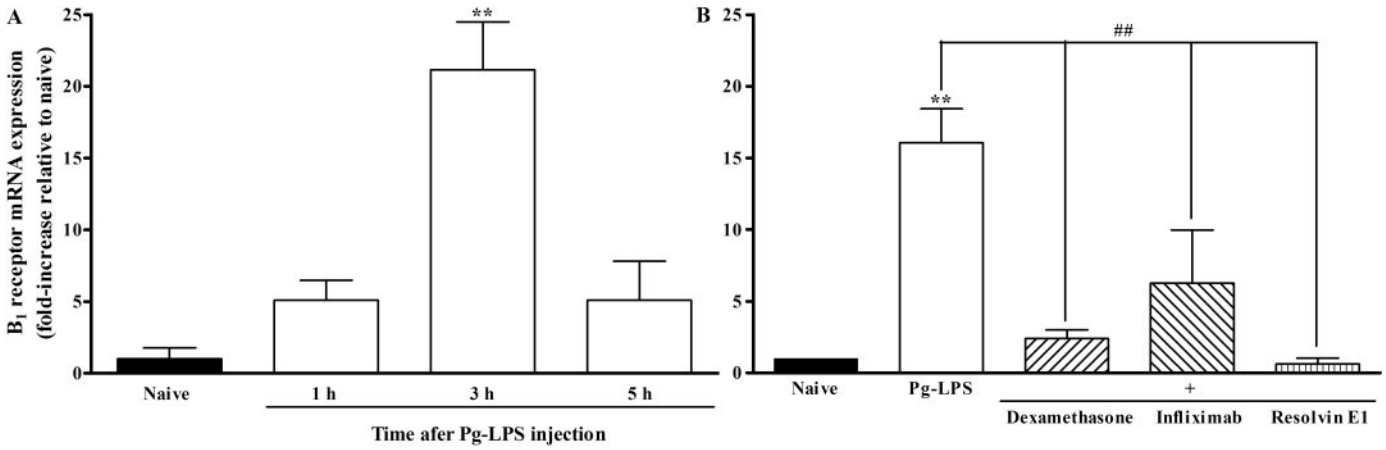

Fig. 3. Effects of Pg-LPS on kinin B_1 receptor mRNA expression. A, time-related effect of Pg-LPS (3 μg/paw) injection on B_1 receptor mRNA expression in the rat paw. B, effects of treatment with: dexamethasone (0.5 mg/kg s.c., 2 h), infliximab (1 mg/kg s.c., 15 min), or resolvin E1 (300 ng/rat i.p., 30 min) on B_1 receptor mRNA expression in rats pretreated with Pg-LPS (3 μg/paw, 3 h). Naive indicates no previous treatment with Pg-LPS. Each column represents the mean of three independent experiments, and the vertical lines are the S.E.M. **, $p < 0.01$, significantly different from control paws; ##, $p < 0.01$, significantly different from LPS-injected paws.

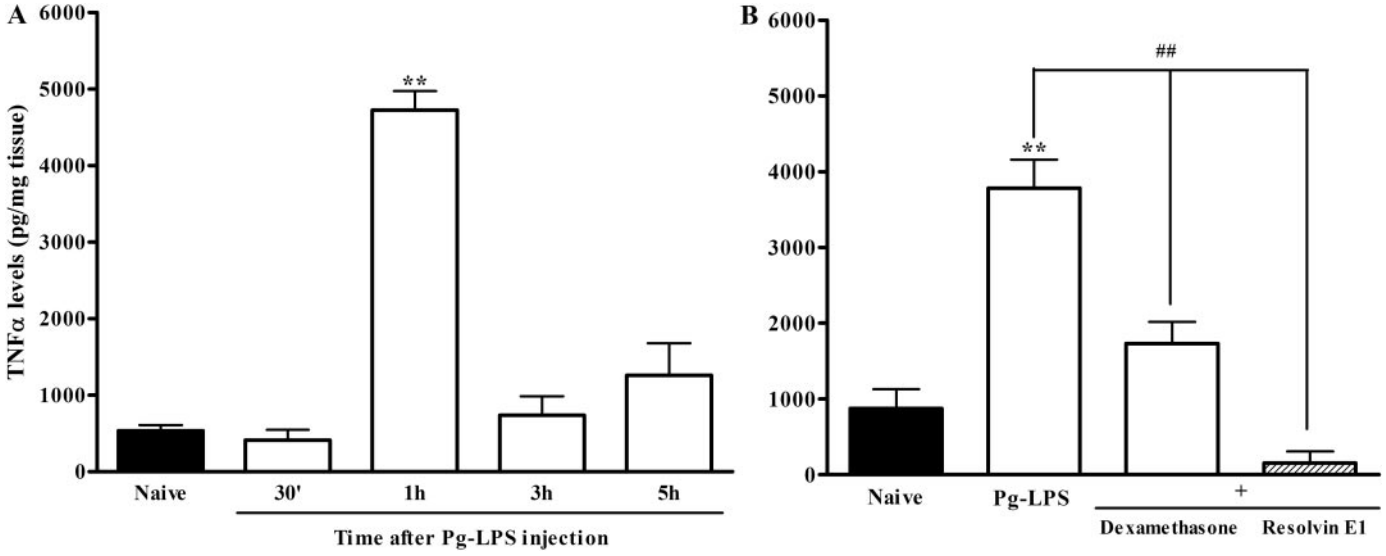

Fig. 4. Effects of Pg-LPS on TNF-α levels (expressed as picograms per milligram tissue) in the rat paw. A, time-related effect of Pg-LPS (3 μg/paw) injection on TNF-α levels in the rat paw. B, effects of treatment with dexamethasone (0.5 mg/kg s.c., 2 h) or resolvin E1 (300 ng/rat i.p., 30 min) on TNF-α levels in animals pretreated with Pg-LPS (3 μg/paw, 1 h). Naive indicates no previous treatment with Pg-LPS. Each point represents the mean ± S.E.M. of five animals. **, $p < 0.01$, significantly different from control paws; ##, $p < 0.01$, significantly different from LPS-injected paws.

ity assay. As shown in Fig. 5A, Pg-LPS injection (1–36 h) was capable of inducing a time-related increase in MPO levels, reaching the maximal values between 3 and 12 h, with an approximately 2-fold augmentation compared with the control group. The increase in MPO activity at 3 h was significantly reduced by the pretreatment with dexamethasone (0.5 mg/kg s.c., 2 h before Pg-LPS) and resolvin E1 (300 ng/animal, 30 min before Pg-LPS) The inhibition indexes for these treatments were 45 ± 6% and 55 ± 3%, respectively. However, this inflammatory parameter was not significantly modified by treatment with infliximab (1 mg/kg s.c., 15 min before Pg-LPS) (Fig. 5B).

Discussion

Kinin B_1 receptors are atypical G-protein-coupled receptors that are not constitutive in general, because they are highly up-regulated after stressful stimuli (Calixto et al., 2004). The induction of this receptor has been associated with generation of inflammatory cytokines, neutrophil migration, and activation of several signaling pathways (Passos et al., 2004; Medeiros et al., 2007). Compelling in vivo and in vitro evidence indicates that kinin B_1 receptors can be up-regulated by infectious stimuli (Calixto et al., 2004; Campos et al., 2006). A few reports have linked periodontal pathogens and kinin B_2 receptors (Griesbacher et al., 1994; Rubinstein et al., 2001; Hu et al., 2006; Brechter et al., 2008). Nevertheless, there is no evidence showing whether Pg-LPS might lead to the up-regulation of B_1 receptors. Our study indicates, for the first time, that B_1 receptors can be up-regulated after in vivo administration of Pg-LPS by mechanisms sensitive to the proresolving mediator resolvin E1.

Previous data from our group demonstrated that local treatment with *E. coli* LPS resulted in a functional up-regulation of B_1 receptors in the rat paw, as assessed by an increase in des-Arg^9-BK-induced edema (Passos et al., 2004).

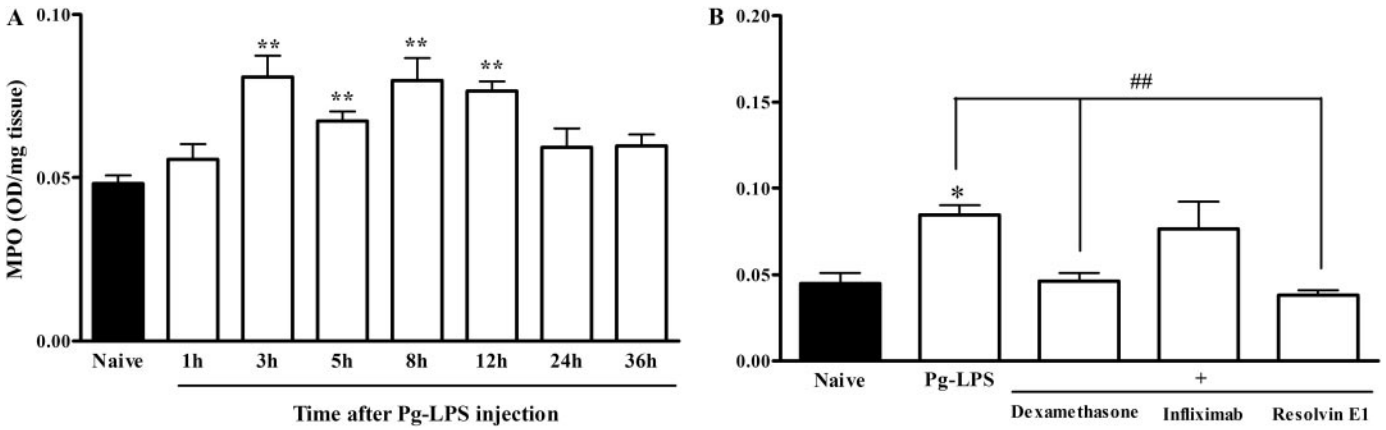

Fig. 5. Effects of Pg-LPS on MPO activity (expressed as OD/mg tissue) in the rat paw. A, time-related effect of Pg-LPS (3 μg/paw) on MPO levels in the rat paw; LPS was injected 1 to 36 h before. B, effects of treatment with dexamethasone (0.5 mg/kg s.c., 2 h), infliximab (1 mg/kg s.c., 15 min), or resolvin E1 (300 ng/rat i.p., 30 min) on neutrophil migration in animals pretreated with Pg-LPS (3 μg/paw, 3 h). Naive indicates no previous treatment with Pg-LPS. Each point represents the mean ± S.E.M. of five animals. *, $p < 0.05$; **, $p < 0.01$, significantly different from control paws; ##, $p < 0.01$, significantly different from LPS-injected paws. OD, optical density.

In this publication, des-Arg^9-BK-caused edema peaked at 12 h, and then decreased gradually between 24 and 36 h. The present results show that Pg-LPS can induce a marked increase in the rat paw edema elicited by des-Arg^9-BK, an effect that was significant between h and 36 h. Literature data have suggested that LPS from *E. coli* preferentially activates TLR4, whereas Pg-LPS displays greater affinity for TLR2 receptors (Darveau et al., 2004; Muthukuru et al., 2005; Kikkert et al., 2007). Furthermore, some publications have pointed to the activation of differential signaling pathways after TLR2 and TLR4 stimulation (Burns et al., 2006; Zhou et al., 2007; Hajishengallis et al., 2008). Concerning B_1 receptors, it seems that both Pg-LPS and *E. coli* LPS are able to produce their up-regulation, although slight temporal and intensity differences are observed.

Dexamethasone is a glucocorticoid that displays several anti-inflammatory actions via genomic and nongenomic means, and it might block TLR signaling by multiple mechanisms (Chinenov and Rogatsky, 2007; Stahn and Buttgereit, 2008). Several studies have demonstrated that dexamethasone is able to block B_1 receptor up-regulation induced by infectious stimuli (Calixto et al., 2004, Passos et al., 2004). Our data show that dexamethasone markedly reduced des-Arg^9-BK-induced edema after Pg-LPS treatment, suggesting that B_1 receptor up-regulation is probably related to de novo protein synthesis. This idea was further confirmed by results showing that functional up-regulation of B_1 receptors was preceded by an increase of B_1 receptor mRNA expression (maximal at 3 h), which was markedly prevented by dexamethasone.

The chimeric anti-TNF-α antibody infliximab produced a partial, but significant inhibition of edema induced by des-Arg^9-BK in Pg-LPS-pretreated rats. This suggests that B_1 receptor functional up-regulation by Pg-LPS is a process that depends, at least in part, on the release of TNF-α. The relevance of TNF-α for up-regulation of B_1 receptors has been also demonstrated elsewhere (Passos et al., 2004; Rocha et al., 2006). Extending our in vivo data, the increased B_1 receptor mRNA expression after Pg-LPS treatment was sensitive to infliximab administration. Relevantly, it was demonstrated that TLR2 activation by Pg-LPS leads to TNF-α up-regulation by the stimulation of transduction pathways distinct from that of *E. coli* LPS; whereas TLR2/JNK is the main pathway for Pg-LPS, the induction by *E. coli* LPS is mediated by TLR4/NF-κB/p38MAPK pathways (Zhang et al., 2008).

It has been demonstrated that resolvin E1 orchestrates the resolution of inflammation by promoting tissue homeostasis (Serhan et al., 2008). As reviewed recently (Serhan et al., 2008; Van Dyke, 2008), resolvin E1 prevents neutrophil infiltration and stimulates the phagocytic activity of macrophages, increasing the exit of inflammatory cells from the inflamed site. Previous results on a rabbit model of periodontitis demonstrated an important role for resolvin E1 in the inhibition of alveolar bone resorption (Hasturk et al., 2007). A recent in vitro study demonstrated that resolvin E1 is able to limit osteoclast growth and bone resorption (Herrera et al., 2008). In our article, we describe further mechanisms for resolvin E1 actions in the responses evoked by periodontal pathogens. We demonstrate, for the first time, the ability of resolvin E1 in reducing des-Arg^9-BK-evoked paw edema in animals pretreated with Pg-LPS. In addition, the administration of a single dose of resolvin E1 almost abolished the increase of B_1 receptor mRNA expression elicited by Pg-LPS. It is possible to propose that resolvin E1 prevents the inflammatory responses evoked by the selective B_1 receptor agonist des-Arg^9-BK, mainly by reducing B_1 receptor mRNA expression. Of course, we cannot rule out that resolvin E1 might interfere in other levels of B_1 receptor regulation.

It would be very pertinent to determine whether resolvin E1 is more potent than dexamethasone or infliximab in preventing B_1 receptor up-regulation. Pilot experiments (data not shown) revealed that no clear dose-response effects were observed when additional doses of dexamethasone (2 mg/kg s.c.), infliximab (10 mg/kg s.c.), or resolvin E1 (100 and 500 ng/rat) were tested on B_1 receptor-mediated edema. A lack of dose-related effects for these inhibitors had been shown previously (Campos et al., 1996; Triantafillidis et al., 2005; Haas-Stepleton et al., 2007; Schwab et al., 2007; Seadi Pereira et al., 2009). Therefore, although the results suggest that resolvin E1 is more potent than dexamethasone and infliximab in preventing B_1 receptor up-regulation, a comparison at the level of ID_{50} values is not possible.

Our data show that Pg-LPS injection into the rat paw

resulted in a marked and time-related increase of TNF-α production, which was maximal at 1 h. This temporal profile was very similar to that observed after *E. coli* LPS treatment, where TNF-α levels were maximal after 1 h in a process sensitive to dexamethasone treatment (Passos et al., 2004). In our study, the increase of TNF-α levels in response to Pg-LPS injection was significantly diminished by dexamethasone and resolvin E1. It was demonstrated that resolvin E1 reduced the leukocyte infiltration, and prevented TNF-α gene expression in a mouse model of colitis (Arita et al., 2005). In this context, we believe that part of the inhibitory effects of Resolvin E1 on B_1 receptors rely on the reduction of TNF-α production. However, the treatment with infliximab failed to significantly reduce the increase of TNF-α levels 1 h after Pg-LPS injection, at least in our experimental conditions and according to evaluation by ELISA experiments (data not shown). Thus, infliximab probably prevents B_1 receptor up-regulation by blocking TNF-α binding to its receptor, rather than by regulating its production.

A correlation between neutrophil migration and the up-regulation of B_1 receptors has been demonstrated previously (Passos et al., 2004; Fernandes et al., 2005). Previous data demonstrate that injection *E. coli* LPS into the rat paw induced a time-related increase in MPO levels, which peaked at 12 h and lasted for up to 36 h (Passos et al., 2004). The results provided herein show that Pg-LPS induced a marked and earlier increase of MPO activity, which was found significant at 3 h, and remained increased for up to 12 h. The temporal profile for increased MPO activity by Pg-LPS was consistent with the up-regulation of B_1 receptor-mediated edema, which suggests that neutrophils might provide signals for B_1 receptor modulation. Interestingly, the elevation of MPO activity induced by Pg-LPS was significantly reduced by pretreatment with dexamethasone, although infliximab failed to significantly affect this parameter. It is possible to surmise that TNF-α increase and neutrophil influx independently contribute to the up-regulation of B_1 receptors by Pg-LPS. Our findings extend previous literature data (Fernandes et al., 2005), which demonstrated that TNF-α production and neutrophil migration are independent events leading to B_1 receptor up-regulation by platelet-activating factor in the rat paw.

In our study, resolvin E1 markedly reduced the increase of MPO activity in response to Pg-LPS injection. These results are consistent with previous evidence showing that resolvin E1 is able to prevent neutrophil infiltration (Hasturk et al., 2007; Serhan et al., 2008). It was demonstrated that resolvin E1 rapidly reduces the leukocyte rolling in mouse venules and it was also found effective in preventing transepithelial migration of isolated human neutrophils (Campbell et al., 2007; Dona et al., 2008). It is alluring to propose that the inhibitory effects of resolvin E1 on B_1 receptor modulation are possibly related to the inhibition of neutrophil migration.

Altogether, our data suggest the following sequence of events leading to B_1 receptor up-regulation by Pg-LPS in vivo: there is a rapid increase of TNF-α levels, followed by increased neutrophil migration, leading to the up-regulation of B_1 receptors. Of interest, the proresolution mediator resolvin E1 is able to down-regulate B_1 receptor expression by reducing both TNF-α release and neutrophil influx. These findings shed new light on the mechanisms underlying B_1 receptor modulation, indicating that this receptor subtype might represent a relevant pathway for the inflammatory responses evoked by periodontal pathogens.

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