Cleavage and activation of proteinase-activated receptor-2 on human neutrophils by gingipain-R from *Porphyromonas gingivalis*

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Abstract Gingipain-R, the major arginine-specific proteinase from *Porphyromonas gingivalis*, a causative agent of adult periodontal disease, was found to cleave a model peptide representing the cleavage site of proteinase-activated receptor-2 (PAR-2), a G-protein-coupled receptor found on the surface of neutrophils. The bacterial proteinase was also shown to induce an increase in the intracellular calcium concentration of enzyme-treated neutrophils, most probably due to PAR-2 activation. This response by neutrophils to gingipain-R may be a mechanism for the development of inflammation associated with periodontal disease.

Key words: Proteinase-activated receptor; *Porphyromonas gingivalis*; Neutrophil; Gingipain-R

1. Introduction

Proteinase-activated receptor-2 (PAR-2) is a member of the seven-transmembrane domain receptor family which couple to G-proteins to produce intracellular signals leading to cellular responses [1]. PAR-2 is expressed in a number of tissues, including the gastrointestinal and respiratory tracts and skin, where expression is primarily in the epidermal and smooth muscle cells [1–10]. The receptor is also expressed by human neutrophils and its stimulation leads to neutrophil activation [11], which may contribute to a neutrophil-induced inflammatory response. In order to evaluate the possible role of PAR-2 in inflammation, however, it is necessary to identify physiological and pathophysiological activators of this receptor, after which it will be possible to determine under which conditions activation of PAR-2 on neutrophils is likely to occur.

Cleavage of the PAR-2 extracellular domain after residue arginine-36 produces a new N-terminus, which acts as a tethered ligand that binds to the receptor and leads to its activation. A synthetic receptor agonist peptide (RAP) corresponding to the tethered ligand is also able to activate the receptor. However, the physiological activators of PAR-2 in most tissues have not yet been identified, although trypsin has been suggested to activate PAR-2 under physiological conditions in the gastrointestinal tract [12]. In addition, tryptase may be important in pathological conditions involving mast cell infiltration and/or degranulation [13,14].

Severe adult periodontitis is characterised by an acute inflammatory response with neutrophil infiltration into the gingival tissue, followed by the establishment of a chronic disease state typified by extensive tissue degradation and eventual bone erosion with loss of teeth [15]. It is known that Gram-negative anaerobes are responsible for adult periodontitis and the bacterium *Porphyromonas gingivalis* has been identified as a major pathogen [16–19]. Proteinases produced by this organism are believed to play a critical role in the virulence of the organism [17,20] and thus in the initiation and progression of adult periodontitis.

Two types of cysteine proteinases, present in multiple forms, are responsible for the so-called trypsin-like activity of *P. gingivalis* [21]. Those specific for arginyl-X bonds (50 and 95 kDa) [22] and that for lysyl-X bonds (105 kDa) [23] have been purified and are referred to as gingipain-R and gingipain-K, respectively. The proteolytic activity of these enzymes, which are associated with both the cell surface and the vesicles of the bacterium [21], has been shown to have a profound disruptive effect on host systems, particularly those controlling inflammatory events. In addition, they have also been demonstrated to decrease blood coagulation [24], enhance vascular permeability [25] and activate the complement system [26], thus contributing to the inflammation seen in periodontal disease. Gingipain-R cleaves complement factor C5 to yield C5a-like fragments with neutrophil chemoattractant activity [27]. The local production of these chemotactic anaphylotoxins could be one mechanism for recruiting neutrophils into the inflamed tissue and for their subsequent activation, with consequent degranulation and generation of oxygen radicals at affected sites [15]. Thus, the question to be addressed is whether gingipain-R can act on receptors such as PAR-2, expressed on the surface of neutrophils, and thereby elicit intracellular signals.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), *N*-formyl-Met-Leu-Phe (fMLP), polymyxin B sulfate and L-cysteine were purchased from Sigma (Sydney, Australia). Fura-2-AM was obtained from Molecular Probes (Eugene, OR, USA). Percoll was purchased from Pharmacia (Sydney, Australia). The fluorescence quenched peptides Abz-Leu-Asp-Pro-Arg-Ser-Phe-Leu-Leu-Lys(Dnp)-Asp-OH (PAR-1) and Abz-Ser-Lys-Gly-Arg-

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Abbreviations: PAR-2, proteinase-activated receptor; RAP, receptor agonist peptide; BSA, bovine serum albumin; fMLP, *N*-formyl-Met-Leu-Phe; Abz, aminobenzoic acid; Dnp, dinitrophenol; $[Ca^{2+}]_i$, intracellular calcium concentration

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Ser-Leu-Ile-Gly-Lys(Dnp)-Asp-OH (PAR-2) were synthesised using standard solid-phase 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry with an automated peptide synthesiser (Applied Biosystems, model 432A). They were purified using reverse-phase high performance liquid chromatography and their structure was confirmed by mass spectroscopy.

2.2. Gingipain-R purification

The 50 kDa form of gingipain-R was purified as described previously [22]. Enzyme was activated by treatment with 10 mM L-cysteine at 37°C, for 15 min, in 0.1 M Tris-HCl, 5 mM $CaCl_2$, pH 7.4. Polymyxin B sulfate (100 μg/ml) was routinely added to inhibit any cellular stimulation by bacterial lipopolysaccharides.

2.3. Enzyme assays and measurement of kinetic constants

Experiments were carried out in 0.1 M HEPES, 0.1 M NaCl, 10 mM $CaCl_2$, 0.2% (w/v) polyethylene glycol (M_r 6000), pH 7.4. The substrate solution (0.5 ml) was allowed to equilibrate to 37°C for 10 min, enzyme solution was added, and enzyme activity monitored by continuously measuring the fluorescence ($\lambda_{ex} = 325$ nm; $\lambda_{em} = 414$ nm [10 nm slits]) in a Perkin Elmer LS-50B spectrofluorometer [28].

Lyophilised PAR-1 and PAR-2 peptides were resuspended in dimethylformamide, and the concentration of the stock solution (6.4 mM) determined spectrophotometrically, assuming an absorption coefficient of 10^4 M^{-1} cm^{-1} at 360 nm [28]. Fluorescence of peptide products after exhaustive cleavage by trypsin was found to be proportional to concentration. Accordingly, an increase in fluorescence could be equated to the increase in concentration of the cleaved substrate, allowing determination of the kinetic parameters, K_m and k_{cat} from an analysis of initial velocities obtained at different substrate concentrations.

2.4. Measurement of intracellular Ca^{2+} responses in isolated neutrophils

Neutrophils were isolated by fractionation of freshly drawn human blood from normal volunteers, using Percoll gradients as described previously [29]. Preparations containing more than 95% neutrophils, with eosinophils as the major contaminant, were suspended at a concentration of 10×10^6 cells/ml in extracellular medium (25 mM HEPES, pH 7.3, 121 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 5 mM $CaCl_2$, 6 mM $NaHCO_3$ and 5.5 mM glucose) containing 0.2% w/v BSA.

Neutrophils were loaded with Fura-2 at room temperature as described previously [29]. Prior to fluorescence measurements, cells were centrifuged, resuspended in extracellular medium (without BSA) at a concentration of 3×10^6 cells/ml and then maintained at 37°C in stirred cuvettes throughout the experiment. The ratio of the Fura-2 fluorescence at 510 nm after excitation at 340 and 380 nm was monitored using a Perkin Elmer LS-50B spectrofluorimeter. After initial baseline recording, trypsin, gingipain-R (50 kDa) and agonist peptide (RAP) were added to the cells, and the ratio of the fluorescence at the two excitation wavelengths measured, which is proportional to the $[Ca^{2+}]_i$.

3. Results and discussion

A synthetic peptide, Abz-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys(Dnp)-Asp-OH, which corresponds to the four amino acids on either side of the cleavage site of human PAR-2 (residues 33–41 of human PAR-2), was evaluated for its accuracy as a predictor of receptor cleavage. The Abz group only fluoresces upon release of the Lys(Dnp) quenching group following proteolytic cleavage. Thus, hydrolysis within the PAR-2 activation sequence by a potential receptor activator can initially be assessed in this manner. In order to test this system, cleavage of the peptide was examined with trypsin and thrombin, which are known to be activating and non-activating for the PAR-2 receptor, respectively. It was found that trypsin cleaved the peptide at concentration as low as 0.1 nM, while thrombin was incapable even at significantly higher levels (0.1–100 nM). Trypsin exhibited a high affinity for the peptide as evidenced by a low K_m value (Table 1), and cleaved the peptide efficiently, with a high k_{cat}/K_m value. This cleavage by trypsin, but not thrombin, supports the hypothesis that this peptide may be considered an effective model for determining the potential of a given proteinase to activate PAR-2.

Table 1
Kinetic parameters of the cleavage of a synthetic peptide corresponding to activation sequence of PAR-2

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM s^{-1})
Trypsin	5.5	86	15.6
Gingipain-R	3.0	50.0	16.6

A novel potential pathophysiological activator of PAR-2, gingipain-R, was then examined for its ability to hydrolyse the PAR-2 peptide, prior to its being tested in cellular systems. Gingipain-R at concentrations as low as 0.1 nM cleaved the test peptide with very similar kinetics to trypsin (Table 1), indicating that the enzyme has the potential to cleave within the receptor itself. The specificity of gingipain-R for the PAR-2 peptide was further indicated by its inability to cleave the peptide representing the activation sequence of the related receptor, PAR-1, Abz-Leu-Asp-Pro-Arg-Ser-Phe-Leu-Leu-Lys(Dnp)-Asp-OH, using concentrations of enzyme of 1–50 nM, despite the fact that the peptide also contains an arginine residue. This is consistent with previous results which showed that gingipain-R was unable to activate PAR-1 on platelets [30].

Cleavage of the synthetic PAR-2 peptide by trypsin and gingipain-R proceeded according to similar kinetic constants, suggesting that cell-surface PAR-2 may be cleaved by gingipain-R. We, therefore, addressed this question by monitoring the increase in intracellular calcium levels of neutrophils in

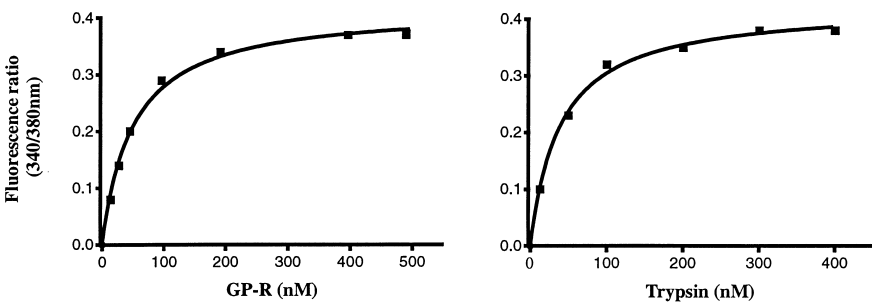

Fig. 1. The $[Ca^{2+}]_i$ response of neutrophils to different concentrations of gingipain-R and trypsin. Neutrophils (from donor MB) were loaded with Fura-2 and stimulated with gingipain-R and trypsin. The increases in $[Ca^{2+}]_i$ at different proteinase concentrations represent the mean ± S.E.M. derived from three traces similar to those shown in Fig. 2.

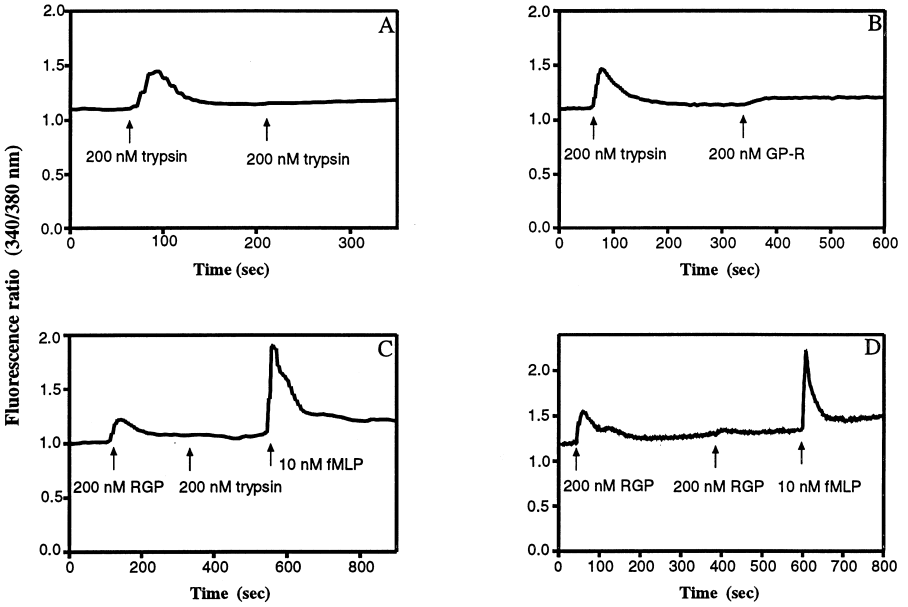

Fig. 2. Effects of desensitisation on $[Ca^{2+}]_i$ responses in neutrophils. Cells were exposed to (A) 200 nM trypsin, then challenged with 200 nM trypsin, (B) 200 nM trypsin, then challenged with 200 nM gingipain-R, (C) 200 nM gingipain-R, then challenged with 200 nM trypsin and (D) 200 nM gingipain-R, then challenged with 200 nM gingipain-R and followed by 10 nM fMLP peptide. This figure shows the desensitisation effects for one of the eight donors in whom these effects were observed.

response to gingipain-R. Neutrophils are a good model for examining this process as they play a role in adult periodontitis and PAR-2 is the only proteinase-activated receptor known to be expressed on the surface of these phagocytic cells [29].

Neutrophils from several donors were individually tested for a $[Ca^{2+}]_i$ response to gingipain-R, trypsin and the receptor agonist peptide (RAP) (Table 2). All samples showed an increase in $[Ca^{2+}]_i$ in response to RAP treatment. The magnitude of the $[Ca^{2+}]_i$ response varied between individuals, confirming results described elsewhere [11], where it was observed that the neutrophils of all donors express PAR-2, including some that did not respond to trypsin.

Gingipain-R induced a dose-dependent increase in $[Ca^{2+}]_i$ in neutrophils. The concentration dependence of such an increase using neutrophils from a single donor is shown in Fig. 1, yielding an enzyme concentration inducing half the maximal response (EC_{50}) of 49.8 ± 3.9 nM and 39.2 ± 3.9 nM for receptor activation by gingipain-R and trypsin, respectively. As with the RAP activation, the magnitude of the $[Ca^{2+}]_i$ increase caused by gingipain-R varied between donors, but the bacterial enzyme induced an increase in $[Ca^{2+}]_i$ only in neutrophils from donors that, in parallel, also exhibited a trypsin-induced increase in intracellular calcium (eight of the 12 donors) (Table 2). The variation in response and the lack of response to enzyme activation in certain donors is again in agreement with previous observations [11]. Neutrophils which did not respond to either proteinase did respond to the fMLP peptide, suggesting that G-protein-coupled receptor signalling pathways in otherwise non-responsive neutrophils were intact.

Proteinase-activated receptors undergo rapid desensitisation after a short period of activation by an enzyme agonist. Since exposure of cells to trypsin desensitises $[Ca^{2+}]_i$ responses to subsequent challenges with trypsin due to cleavage of PAR-2 (Fig. 2A) [31], experiments were carried out using the $[Ca^{2+}]_i$ mobilisation assay in order to determine if gingipain-R and

Table 2
Intracellular calcium flux in response to various agonists in neutrophils from 12 donors

Donor	Fluorescence ratio 340/380 nm			
	GP-R 200 nM	Trypsin 200 nM	RAP 300 μM	fMLP 10 nM
1	0.50	0.41	0.45	0.80
2	0.37	0.35	0.40	0.87
3	0.30	0.12	0.42	1.39
4	0.23	0.28	0.32	0.50
5	0.22	0.18	0.28	0.75
6	0.18	0.12	0.48	0.68
7	0.09	0.10	0.50	0.81
8	0.08	0.13	0.20	0.50
9	0	0	0.23	1.05
10	0	0	nd	0.50
11	0	0	nd	0.30
12	0	0	nd	0.50

nd, not determined.

trypsin were activating the same receptor. When neutrophils were initially activated with gingipain-R, a secondary response to this enzyme was virtually eliminated (Fig. 2D). Exposure of neutrophils to trypsin also desensitised the $[Ca^{2+}]_i$ response to a second challenge with gingipain-R (Fig. 2B). Similarly, exposure of neutrophils to gingipain-R desensitised the response to a second challenge by trypsin (Fig. 2C). The fMLP peptide caused an increase in $[Ca^{2+}]_i$ in gingipain-R-treated neutrophils (Fig. 2D), indicating that depletion of intracellular calcium is not the mechanism of desensitisation. Since PAR-2 is a known proteinase-activated receptor on neutrophils which can be cleaved by trypsin [15,29], these results strongly suggest that trypsin and gingipain-R activate a common receptor on neutrophils, i.e. PAR-2.

It has been shown that stimulation of PAR-2 by RAP leads to neutrophil activation as indicated by an increase in the expression of CD11b and shape changes [11]. PAR-2 activation in a human keratinocyte cell line has been shown to induce the expression of granulocyte-macrophage colony-stimulating factor and interleukin-6, both factors which are known to participate in inflammatory processes [32]. Neutrophil activation by gingipain-R acting via PAR-2 may therefore exacerbate the inflammation seen in periodontal disease and thus the results presented here raise the possibility that *P. gingivalis*, through its proteinase gingipain-R, makes use of host cell receptors to exacerbate bacterially induced tissue destruction.

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