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Mechanisms Mediating *Porphyromonas gingivalis* Gingipain RgpA-Induced Oral Mucosa Inflammation In Vivo

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Suffusion of gingipain RgpA (GRgpA) elicited a significant concentration-dependent increase in the clearance of macromolecules from in situ hamster cheek pouch which was attenuated by NPC 17647, a selective bradykinin B_2 receptor antagonist. Leupeptin and a mixture of proteinase inhibitors also attenuated GRgpA-induced responses. These data indicate that GRgpA elicits plasma exudation from in situ oral mucosa in a catalytic site-dependent fashion by elaborating bradykinin.

A large body of experimental evidence suggests that *Porphyromonas gingivalis*, an oral anaerobic bacterium, plays a role in the pathogenesis of adult periodontitis (2, 9, 13–16, 32). A cardinal manifestation of this condition is plasma exudation from postcapillary venules (11, 32). However, the mechanisms underlying this response are uncertain (1, 4, 5, 7, 14, 15, 27). To this end, gingipain RgpA, a potent arginine-specific cysteine proteinase produced by *P. gingivalis* (2, 12, 16), has been shown to activate the kallikrein/kinin metabolic pathway in guinea pig skin to release bradykinin, a potent 9-amino-acid phlogistic mediator (1, 7, 33), thereby leading to plasma exudation (14, 15). The purpose of this study was to determine whether gingipain RgpA elicits plasma exudation from in situ oral mucosa by elaborating bradykinin.

To visualize the microcirculation and determine the clearance of macromolecules from in situ hamster cheek pouch, we used a method previously described by our laboratory and by other investigators (5–8, 18, 21, 23, 24, 26, 30, 33). Briefly, pentobarbital sodium-anesthetized, tracheostomized, and spontaneously breathing adult male golden Syrian hamsters were used (128 ± 2 g of body weight, $n = 42$). Cheek pouch microcirculation was visualized with a fluorescence microscope (magnification, ×40). Macromolecular leakage was determined by extravasation of fluorescein isothiocyanate-labeled dextran (FITC-dextran; molecular mass, 70 kDa), an intravascular tracer, which appeared as fluorescent spots or leaky sites around postcapillary venules. The number of leaky sites was counted in three random microscopic fields, averaged, and expressed as the number of leaky sites per 0.11 cm^2 of cheek pouch, which corresponds to the area of one microscopic field (5–8, 18, 26, 33). The concentration of FITC-dextran in the plasma and suffusate was determined from a standard curve of FITC-dextran concentration versus percent emission using a spectrophotofluorometer. Clearance of FITC-dextran was determined by calculating the ratio of suffusate (ng/ml) to plasma (mg/ml) concentration of FITC-dextran and multiplying this ratio by the suffusate flow rate (2 ml/min).

The experimental design has been previously used in studies in our laboratory and in others (5–8, 26, 33). Gingipain RgpA was activated with NaOH-neutralized cysteine (20 mM) at 30°C for 15 min before each experiment (14, 15). After suffusing the buffer on the cheek pouch for 30 min (equilibration period), FITC-dextran was injected intravenously (i.v.) and the number of leaky sites and the clearance of FITC-dextran were determined for 30 min. Then two concentrations of gingipain RgpA (0.001 and 0.01 μM) were suffused in random order for 10 min each. The number of leaky sites was determined every minute for 15 min and at 5-min intervals for 60 min thereafter. The clearance of FITC-dextran was determined before and every 5 min after the start of gingipain RgpA suffusion for 60 min. The time interval between subsequent suffusions of gingipain RgpA was at least 45 min. In another group of animals, NPC 17647 (1.0 μM), a selective bradykinin B_2 receptor antagonist (1, 7, 27), was suffused on the cheek pouch for 30 min before suffusion of gingipain RgpA (0.001 or 0.01 μM). In another series of experiments, leupeptin, a predominantly cysteine proteinase inhibitor that inactivates gingipain RgpA (2, 14, 15), or a mixture of proteinase inhibitors composed of aprotinin (5 μg/ml), soybean trypsin inhibitor (100 μg/ml), bestatin (10 μM), and DL-2-mercapto-methyl-3-guanidinoethylthiopropanoic acid (10 μM) to inhibit serine proteinases, including kallikrein, aminopeptidases, and carboxypeptidase N, was suffused on the cheek pouch for 30 min before suffusion of gingipain RgpA (0.001 or 0.01 μM). Lastly, gingipain RgpA (0.001 or 0.01 μM) was suffused for 10 min before and after i.v. infusion of indomethacin (10 mg/kg of body weight) over a 30-min period. The concentrations of gingipain RgpA, NPC 17647, leupeptin, and indomethacin used in these experiments were based on previous studies in our laboratory and reports in the literature (2, 3, 7, 14, 15, 23, 25, 26, 28, 33). The concentration of indomethacin used has been shown to inhibit cyclooxygenase in the cheek pouch (23, 25). The composition of the mixture of proteinase inhibitors and the concentrations of its constituents used in these studies were based on previous stud-

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ies in our laboratory and reports in the literature (3, 7, 17, 20, 22, 29).

Purified gingipain RgpA (14 μM) was prepared in Tris buffer (pH 6.8) as previously described (2). FITC-dextran, cysteine, aprotinin, soybean trypsin inhibitor, and indomethacin were obtained from Sigma Chemical Co. Leupeptin and bestatin were obtained from Peninsula Laboratories. DL-2-Mercapto-methyl-3-guanidinoethylthiopropanoic acid was obtained from Calbiochem-Novabiochem Corp. NPC 17647 was a gift from Nova Pharmaceutical Corporation. Indomethacin was dissolved in 5% Na_2CO_3. All other drugs were dissolved in saline. Drugs were freshly prepared before each experiment and were diluted in saline to the desired concentrations.

When a test compound was suffused over the cheek pouch, we determined the maximal change in the number of leaky sites and the clearance of FITC-dextran and used those data as the response to that compound. Data are expressed as means ± standard errors of the means, except for body weight, which is expressed as mean ± standard deviation. Statistical analysis was performed using two-way analysis of variance and the Newman-Keuls test for multiple comparisons. A P value of <0.05 was considered significant.

Suffusion of saline (vehicle) alone for the entire duration of the experiment evoked no visible leaky site formation or significant increase in the clearance of FITC-dextran from the baseline ($15 \times 10^{-6} \pm 2 \times 10^{-6}$ ml/min at the beginning and $16 \times 10^{-6} \pm 3 \times 10^{-6}$ ml/min at the conclusion of the experiment, respectively; for each group, $n = 4$, $P > 0.5$). Likewise, suffusion of NaOH-neutralized cysteine (20 mM) alone for 10 min elicited no visible leaky site formation or significant increase in clearance of FITC-dextran from the baseline ($n = 4$, $P > 0.5$). Repeated suffusions of gingipain RgpA (0.001 and 0.01 μM) for 10 min each with 45-min suffusion of saline in between were associated with significant and reproducible leaky site formation (3 ± 1 and 8 ± 1 per 0.11 cm^2 and 4 ± 2 and 8 ± 2 per 0.11 cm^2, respectively; for each group, $n = 4$, $P < 0.05$ in comparison to saline, and $P > 0.5$ within group comparisons) and an increase in the clearance of FITC-dextran ($23 \times 10^{-6} \pm 3 \times 10^{-6}$ ml/min and $33 \times 10^{-6} \pm 4 \times 10^{-6}$ ml/min, and $22 \times 10^{-6} \pm 4 \times 10^{-6}$ ml/min and $34 \times 10^{-6} \pm 3 \times 10^{-6}$ ml/min, respectively; for each group, $n = 4$, $P < 0.05$ in comparison to saline, and $P > 0.5$ within group comparisons) (Table 1). Leaky site formation was visible within 6 to 7 min after the start of suffusion and was maximal 3 to 4 min after suffusion of gingipain was stopped. The number of leaky sites and clearance of FITC-dextran returned to the baseline 20 min after suffusion of gingipain RgpA was stopped.

Repeated suffusions of NPC 17647 (1.0 μM) alone for 30 min evoked no visible leaky site formation or significant increase in the clearance of FITC-dextran from the baseline and in comparison to saline ($n = 4$, $P > 0.5$). Suffusion of NPC 17647 (1.0 μM, 30 min) significantly attenuated gingipain RgpA (0.001 and 0.01 μM)-induced responses (Table 1) ($P < 0.05$). Repeated suffusions of leupeptin (1.0 μM) alone for 30 min elicited no visible leaky site formation or significant increase in the clearance of FITC-dextran from the baseline ($n = 4$, $P > 0.5$). Leupeptin (1.0 μM) significantly attenuated gingipain RgpA (0.001 and 0.01 μM)-induced leaky site formation and increase in the clearance of FITC-dextran (Table 1) ($P < 0.05$). Likewise, repeated suffusions of the mixture of proteinase inhibitors consisting of aprotinin (5 μg/ml), soybean trypsin inhibitor (100 μg/ml), bestatin (10 μM), and DL-2-mercapto-methyl-3-guanidinoethylthiopropanoic acid (10 μM) alone for 30 min elicited no visible leaky site formation or significant increase in the clearance of FITC-dextran from the baseline ($n = 4$, $P > 0.5$). This mixture significantly attenuated gingipain RgpA (0.001 and 0.01 μM)-induced responses (Table 1) ($P < 0.05$). Intravenous infusion of indomethacin (10 mg/kg) alone for 30 min evoked no visible leaky site formation or significant increase in the clearance of FITC-dextran from the baseline ($n = 4$, $P > 0.5$). Indomethacin significantly potentiated leaky site formation and clearance of FITC-dextran elicited by a low (0.001 μM) but not a high (0.01 μM) concentration of gingipain RgpA (Table 1) ($P < 0.05$).

TABLE 1. Effects of gingipain RgpA on leaky site formation and clearance of FITC-dextran from in situ hamster cheek pouch[a]

Intervention mixture	Leaky sites (no. per 0.11 cm^2)	Clearance (ml/min, 10^{-6})
Saline (control)	Nil	15 ± 2
GRgpA (0.001 μM)	3 ± 1*	23 ± 3*
GRgpA (0.001 μM) and NPC 17647 (1 μM)	Nil†	15 ± 4†
GRgpA (0.01 μM)	8 ± 1*	33 ± 4*
GRgpA (0.01 μM) and NPC 17647	2 ± 1†	13 ± 3†
GRgpA (0.001 μM) and leupeptin (1 μM)	Nil†	13 ± 3†
GRgpA (0.01 μM) and leupeptin (1 μM)	1 ± 1†	13 ± 2†
GRgpA (0.001 μM) and PI mixture	Nil†	13 ± 4†
GRgpA (0.01 μM) and PI mixture	1 ± 1†	18 ± 7†
GRgpA (0.001 μM) and indomethacin (10 mg/ml)	6 ± 1†	42 ± 6†
GRgpA (0.01 μM) and indomethacin (10 mg/ml)	9 ± 1†	39 ± 5†

[a] Values are means ± standard errors of the means; for each group, $n = 4$ animals. GRgpA, gingipain RgpA; PI mixture, mixture of proteinase inhibitors consisting of aprotinin (5 μg/ml), soybean trypsin inhibitor (100 μg/ml), bestatin (10 μM), and DL-2-mercapto-methyl-3-guanidinoethylthiopropanoic acid (10 μM). An asterisk indicates a P value of <0.05 in comparison to saline (control). A dagger represents a P value of <0.05 in comparison to gingipain RgpA alone. Nil, no leaky sites were detected.

There are three new findings of this study. Firstly, we found that suffusion of gingipain RgpA increases macromolecular efflux from in situ hamster cheek pouch, an animal model whose value for studying the effects of proinflammatory mediators on plasma exudation from in situ oral mucosa is well established (5–8, 18, 19, 21, 23, 24, 26, 30, 31, 33). This response was mediated by local production of bradykinin, because NPC 17647, a selective bradykinin B_2 receptor antagonist, significantly attenuated gingipain RgpA-induced responses. Moreover, a mixture of proteinase inhibitors, some of which have been shown to inhibit several steps in the kallikrein/kinin metabolic pathway but not gingipain RgpA activity (1, 12, 14–17, 20, 22), abrogated gingipain RgpA-induced responses. These data support and extend previous studies in the literature that showed that exogenous gingipains activate the kallikrein/kinin metabolic pathway in guinea pig skin and elicit plasma exudation (10, 14–16).

Secondly, leupeptin, a predominantly cysteine proteinase inhibitor that inactivates gingipain RgpA and abrogates its edema-forming effects in guinea pig skin (2, 14, 15), circumvented the increase in macromolecular efflux evoked by gingipain RgpA. This observation implies that the edema-forming effects of gingipain RgpA are catalytic site dependent. Lastly, indomethacin, at a concentration that inhibits cyclooxygenase in the cheek pouch (23, 25), amplified macromolecular efflux elicited by a low (submaximal) concentration of gingipain

RgpA. These data suggest that at low concentrations gingipain RgpA stimulates the production of anti-inflammatory prostaglandins in the cheek pouch that partly counteract the edema-forming effects of the proteinase (23, 25). However, at high concentrations of gingipain RgpA the salutary effects of these compounds are circumvented, in part, by local elaboration of bradykinin. Collectively, these data indicate that gingipain RgpA increases macromolecular efflux from in situ oral mucosa in a catalytic site-dependent fashion through local elaboration of bradykinin.

The mechanisms underlying gingipain RgpA-induced bradykinin and prostaglandin production in in situ hamster cheek pouch were not elucidated in this study. Nonetheless, the results of this study unravel a novel mechanism whereby gingipain RgpA could injure the gingiva in susceptible individuals (2, 9–11, 13–15, 32). We propose that upon release by *P. gingivalis*, gingipain RgpA activates the kallikrein/kinin metabolic pathway in gingival mucosa to produce bradykinin, leading to plasma exudation and tissue injury (1, 7, 11, 13, 32). This cascade of biologic responses may be amplified by other proteinases released by oral keratinocytes, which are the first cells in the oral mucosa exposed to gingipain RgpA (26). Whether gingipain RgpA activates the kallikrein/kinin metabolic pathway in the oral mucosa directly or stimulates oral keratinocytes to release bradykinin-forming proteinases remains to be determined.

In summary, we found that gingipain RgpA increases macromolecular efflux from in situ hamster cheek pouch in a catalytic site-dependent fashion through local elaboration of bradykinin. Full expression of the edema-forming effects of gingipain RgpA is circumvented, in part, by concomitant production of anti-inflammatory prostaglandins. We suggest that topical application of bradykinin B_2 receptor antagonists could be useful in the treatment of adult periodontitis.

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