Subtilisin Increases Macromolecular Efflux from the Oral Mucosa

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The purpose of this study was to determine whether subtilisin, a potent serine proteinase derived from Bacillus species contaminating smokeless tobacco, increases macromolecular efflux from the oral mucosa and, if so, whether local elaboration of bradykinin mediates this response. Using intravitral microscopy, I found that subtilisin increases macromolecular efflux from the in situ hamster cheek pouch (P < 0.05). Heat-inactivated subtilisin had no significant effects on macromolecular efflux. Subtilisin-induced responses were significantly attenuated by Hoe 140 and NPC 17647, two structurally distinct selective bradykinin B₂ receptor antagonists, but not by des-Arg⁹-[Leu⁸]bradykinin, a selective bradykinin B₁ receptor antagonist, or CP-96,345, a selective neurokinin-1 receptor antagonist. Aprotinin, but not leupeptin, significantly attenuated subtilisin-induced increase in macromolecular efflux. Indomethacin had no significant effects on subtilisin-induced responses. Collectively, these data indicate that subtilisin increases the macromolecular efflux from the in situ hamster cheek pouch in a catalytic-site-dependent fashion through local elaboration of bradykinin. This response does not involve the stimulation of local afferent nerves or the production of prostaglandins.

A growing body of clinical evidence suggests that the regular use of smokeless tobacco is associated with oral mucosa injury and inflammation in humans (3, 11, 32, 35). A cardinal feature of this response is plasma exudation from postcapillary venules that leads to interstitial edema and tissue dysfunction (7, 11). Although the mechanisms underlying smokeless-tobacco-induced plasma exudation from the oral mucosa are uncertain, previous work from my laboratory has established that local elaboration of bradykinin, a potent phlogistic 9-amino-acid peptide released from kininogen (1, 4, 38), mediates a smokeless-tobacco-induced increase in macromolecular efflux. However, the nature of the putative factor(s) in smokeless tobacco that activates the kallikrein/kinin metabolic pathway in the oral mucosa to release bradykinin is uncertain (1, 12, 19, 25).

To this end, spore-producing Bacillus species which elaborate subtilisin, a potent serine proteinase that activates the kallikrein/kinin system (1, 13, 17, 19, 29), have been shown to contaminate tobacco leaves used to prepare smokeless tobacco for commercial use (34). Once smokeless tobacco is placed on the oral mucosa, the local microenvironment is conducive for the sporulation of Bacillus species and the release of subtilisin (7, 13, 17, 21, 29, 34, 38). Whether subtilisin thus released activates the kallikrein/kinin system and evokes plasma exudation from the in situ oral mucosa is uncertain.

Hence, the purpose of this study was to begin to address this issue by determining whether subtilisin increases the macromolecular efflux from the in situ hamster cheek pouch and, if so, whether local elaboration of bradykinin mediates this response.

MATERIALS AND METHODS

General methods. (i) Preparation of animals. Adult, male golden Syrian hamsters weighing 132 ± 2 g (n = 36) were anesthetized with pentobarbital sodium (6 mg/100 g [body weight]) given intraperitoneally. A tracheotomy was performed to facilitate spontaneous breathing. A femoral vein was cannulated to inject the intravascular tracer, fluorescein isothiocyanate-labeled dextran (FITC-dextran; molecular mass, 70 kDa), and supplemental anesthesia (2 to 4 mg/100 g [body weight]/h). A femoral artery was cannulated to obtain arterial blood samples and to monitor the arterial blood pressure, which did not change significantly during the experiments. Body temperature was kept constant (37 to 38°C) throughout the experiment by using a heating pad.

To visualize the microcirculation of the cheek pouch, I used a method previously used in my laboratory and by other investigators (5–10, 14, 20, 24, 26, 28, 33, 38). Briefly, the left cheek pouch was washed gently over a small plastic baseplate, and an incision was made in the outer skin to expose the cheek pouch membrane. The vascular connective tissue layer was removed and a plastic chamber was positioned over the baseplate and secured in place by suturing the skin around the upper chamber. This chamber contains the diffusion fluid. This arrangement forms a triple-layered complex, the baseplate, the upper chamber, and the cheek pouch membrane exposed between the two plates. After these initial procedures, the hamster was transferred to a heated microscope stage. The chamber was connected to a reservoir containing warmed bicarbonate buffer (37 to 38°C), which allowed continuous suffusion of the cheek pouch. The buffer was bubbled continuously with 95% N₂–5% CO₂ (pH 7.4). The chamber was also connected via a three-way valve to an infusion pump (model 314B; Sage Instruments, Boston, Mass.) that allowed constant administration of subtilisin and drugs into the suffusate.

(ii) Determination of clearance of macromolecules. The cheek pouch microcirculation was visualized with an Olympus microscope (Jacobs Instruments, Shawnee Mission, Kans.) coupled to a 100-W mercury light source at a magnification of ×40. Fluorescence microscopy was accomplished with the aid of filters that matched the spectral characteristics of FITC-dextran (7, 14). Macromolecular leakage was determined by extravasation of FITC-dextran, which appeared as fluorescent “spots” or leaky sites around the postcapillary venules. The number of leaky sites was determined by counting three random microscopic fields corresponding to the area of one microscopic field, as previously described in my laboratory (5, 9, 28, 38).

In experiments in which the clearance of FITC-dextran was calculated, the suffusate was collected at 5-min intervals throughout the experiment by a fraction collector (Microfractionator; Gilson Medical Electronics, Middleton, Wis.). Samples were collected in glass test tubes, and the concentration of FITC-
Dextran was determined. Arterial blood samples were collected in heparinized capillary tubes (70-μl volume; Scientific Products, McGaw Park, Ill.), beginning 5 min before and at 5, 30, 60, 120, 180, and 240 min after injection of FITC-dextran. The concentration of FITC-dextran was determined in all plasma samples. Other investigators and I have shown that the plasma concentration of FITC-dextran peaks within 10 min of injection and decreases slowly thereafter during the entire duration of the experiment (14). To quantitate the concentration of FITC-dextran in the plasma and suffusate, a standard curve for FITC-dextran concentrations versus the percent emission was determined with a spectrophotofluorometer (Photon Technology International, Inc., Princeton, N.J.). The standard was FITC-dextran prepared on a weight-per-volume basis. With the bicarbonate buffer used as background, a standard curve was generated for each experiment, and each curve was subjected to linear regression analysis. The percent emission for unknown samples (plasma and suffusate) was measured on the spectrophotofluorometer, and the concentration of FITC-dextran was calculated from the standard curve. In preliminary experiments, minimal fluorescence signal (<2% above background) was detected when drugs were added to the buffer and when plasma and suffusate samples were examined before the addition of FITC-dextran. The clearance of FITC-dextran was determined by calculating the ratio of suffusate concentration (in nanograms per milliliter) to plasma concentration (in milligrams per milliliter) of FITC-dextran and then multiplying this ratio by the suffusate flow rate (2 ml/min) (5–9, 28, 38).

Experimental protocols. (i) Effects of subtilisin on macromolecular efflux. The purpose of this study was to determine whether suffusion of subtilisin elicits leaky site formation and increases the clearance of FITC-dextran from the cheek pouch. After suffusing buffer 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. The concentration of FITC-dextran in the suffusate rose rapidly after the injection and stabilized within 30 min, while no leaky sites were observed. Then, two concentrations of subtilisin (0.1 and 0.5 μM) were suffused in a nonsystematic fashion. Each concentration was suffused for 10 min. The number of leaky sites was determined every minute for 7 min and at 5-min intervals for 60 min thereafter. The clearance of FITC-dextran was determined before and every 5 min after for 60 min. The time interval between subsequent applications of subtilisin was at least 45 min (7, 33).

To test the specificity of subtilisin-induced responses, subtilisin (0.1 and 0.5 μM) was heated at 60°C for 15 min in another series of experiments before being suffused on the cheek pouch. The number of leaky sites and the clearance of FITC-dextran were determined after each intervention. In preliminary studies, I determined that repeated suffusions of subtilisin (0.1 and 0.5 μM) for 10 min each were associated with reproducible leaky site formation (16 ± 0.11 and 17 ± 0.11 cm² versus 16 ± 0.11 and 17 ± 0.11 cm², respectively; each group, n = 4; P > 0.5) and an increase in the clearance of FITC-dextran [24 ± 5 × 10⁻⁶ and [23 ± 4] × 10⁻⁶ ml/min versus [52 ± 12] × 10⁻⁶ and [54 ± 11] × 10⁻⁶ ml/min, respectively; each group, n = 4; P > 0.5]. In addition, suffusion of saline (vehicle) for the entire duration of the experiment was associated with no visible leaky site formation or increase in clearance of FITC-dextran (data not shown). The concentrations of subtilisin used in these studies were based on preliminary studies.

(ii) Effects of bradykinin receptor antagonists on subtilisin-induced responses. Gao et al. (7) showed that the edema-forming effects of smokeless tobacco in the in situ cheek pouch are mediated through elaboration of bradykinin and stimulation of bradykinin B₂ receptors (1, 4, 28, 30, 38). The purpose of this study was to determine whether bradykinin B₂ receptor blockade atten-
uates subtilisin-induced leaky site formation and the increase in clearance of FITC-dextran. After the equilibration period, FITC-dextran was injected i.v., and the number of leaky sites and clearance of FITC-dextran were determined as outlined above. Once suffusion of subtilisin was stopped and the number of leaky sites returned to baseline, Hoe 140 or NPC 17647 (each at 1 μM), two selective bradykinin B2 receptor antagonists, or des-Arg9-[Leu8]bradykinin (1 μM), a selective bradykinin B1 receptor antagonist, were suffused for 30 min, and the suffusion of subtilisin was repeated. The number of leaky sites and the clearance of FITC-dextran were determined during each intervention. In preliminary studies, I determined that the suffusion of Hoe 140, NPC 17647, and des-Arg9-[Leu8]bradykinin used in these experiments are based on previous studies in my laboratory (7, 28, 38).

(iii) Effects of a NK1 receptor antagonist on subtilisin-induced responses. The purpose of this study was to determine whether subtilisin-induced responses are mediated, in part, by bradykinin stimulation of afferent nerves to release of substance P (1, 8). To accomplish this goal, I determined whether neurokinin-1 (NK1) receptor blockade, the neurokinin receptor subtype that mediates the substance P-induced increase in macromolecular eflux from the cheek pouch (8), attenuates subtilisin-induced leaky site formation and an increase in clearance of FITC-dextran. After the equilibration period, FITC-dextran was injected i.v., and the number of leaky sites and clearance of FITC-dextran were determined as outlined above. Once suffusion of subtilisin was stopped and the number of leaky sites returned to baseline, CP-96,345 (5 mg/kg), a selective NK1 receptor antagonist, was infused i.v. for 30 min, and suffusion of subtilisin was repeated. The number of leaky sites and the clearance of FITC-dextran were determined during each intervention. In preliminary studies, I determined that i.v. infusion of CP-96,345 (5 mg/kg) alone for 30 min was associated with no visible leaky site formation or increase in clearance of FITC-dextran (data not shown). The concentration of CP-96,345 used in these experiments is based on a previous study in my laboratory (8).

(iv) Effects of aprotinin and leupeptin on subtilisin-induced responses. The purpose of these studies was to determine whether products released through the cyclooxygenase pathway of arachidonic acid metabolism mediate, in part, subtilisin-induced responses. The experimental design was similar to that outlined above except that subtilisin (0.1 and 0.5 μM) was suffused for 10 min before and after the suffusion of aprotinin (5 mg/ml) for 30 min. In another series of experiments, subtilisin (0.1 μM) was suffused for 10 min before and after the suffusion of leupeptin (10 μg/ml) for 30 min. The number of leaky sites and the clearance of FITC-dextran were determined during each intervention. In preliminary studies, I determined that suffusion of aprotinin (5 mg/ml) and leupeptin (10 μg/ml) alone for 30 min was associated with no visible leaky site formation or increase in the clearance of FITC-dextran (data not shown). The concentrations of aprotinin and leupeptin used in these studies were based on previous studies in my laboratory and on reports in the literature (2, 9, 13, 17, 18, 22, 31, 38).

(v) Effects of indomethacin on subtilisin-induced responses. The purpose of these studies was to determine whether products released through the cyclooxygenase pathway of arachidonic acid metabolism mediate, in part, subtilisin-induced responses. The purpose of this study was to determine whether the edema-forming effects of subtilisin are catalytic-site dependent by determining whether aprotinin, a serine proteinase inhibitor, and leupeptin, a predominantly cysteine proteinase inhibitor, attenuate subtilisin-induced responses. The experimental design was similar to that outlined above except that subtilisin (0.1 and 0.5 μM) was suffused for 30 min before and after the suffusion of aprotinin (5 μg/ml) for 30 min. In another series of experiments, subtilisin (0.1 μM) was suffused for 10 min before and after the suffusion of leupeptin (10 μg/ml) for 30 min. The number of leaky sites and the clearance of FITC-dextran were determined during each intervention. In preliminary studies, I determined that suffusion of aprotinin (5 μg/ml) and leupeptin (10 μg/ml) alone for 30 min was associated with no visible leaky site formation or increase in the clearance of FITC-dextran (data not shown). The concentrations of aprotinin and leupeptin used in these studies were based on previous studies in my laboratory and on reports in the literature (2, 9, 13, 17, 18, 22, 31, 38).
induced increase in macromolecular efflux (2, 17, 24). The experimental design was similar to that outlined above except that subtilisin (0.1 and 0.5 mM) was suffused for 10 min before and after indomethacin (10 mg/kg) was infused i.v. over a 30-min period using an infusion pump (final volume, 1 ml). The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, I determined that infusion of indomethacin (10 mg/kg) alone for 30 min was associated with no visible leaky site formation or increase in clearance of FITC-dextran (data not shown). The concentration of indomethacin used in these studies was based on a previous study in my laboratory and has been previously shown to inhibit cyclooxygenase in the cheek pouch (24, 27).

(vi) Drugs. FITC-dextran, subtilisin, des-Arg9-[Leu8]bradykinin, aprotinin, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Leupeptin was obtained from Peninsula Laboratories (Belmont, Calif.). Hoe 140 was a gift from Hoechst Marion Roussel Pharmaceuticals, Inc. (Somerville, N.J.). NPC 17647 was a gift from Nova Pharmaceutical Corporation (Baltimore, Md.). CP-96,345 was a gift from Pfizer Inc. (New York, N.Y.). Indomethacin was dissolved in 5% Na2CO3. All other drugs were dissolved in saline. Drugs were prepared fresh before each experiment and were diluted in saline to the desired concentrations.

(vii) Data and statistical analyses. When a test compound was suffused over the cheek pouch, I determined the maximal change in the number of leaky sites and used it as the response to that compound. Data are expressed as means ± the standard error of the mean (SEM), except for body weight, which is expressed as the mean ± the standard deviation. Because the number of leaky sites returned to baseline (nil) between successive applications of test compounds, all vehicle (saline) control data are expressed as a single value for each experimental condition. 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.

RESULTS

Effects of subtilisin on macromolecular efflux. The suffusion of subtilisin elicited significant, concentration-dependent leaky site formation and an increase in the clearance of FITC-dextran from the cheek pouch (Fig. 1 to 5; each group, n = 4; P < 0.05). Leaky site formation was visible within 6 to 7 min after the start of suffusion and was maximal by 3 to 4 min after the suffusion of subtilisin was stopped. The number of leaky sites and the clearance of FITC-dextran returned to baseline by 20 min after the suffusion of subtilisin was stopped. By contrast, suffusion of heat-inactivated subtilisin (0.1 and 0.5 μM) elicited no visible leaky site formation or increase in the clearance of FITC-dextran relative to saline (vehicle; [15.9 ± 5.3] × 10⁻⁴,

![Figure 3](http://cvl.asm.org/...)}
Effects of bradykinin receptor antagonists on subtilisin-induced responses. Pretreatment with Hoe 140 or NPC 17647 (each at 1 mM) but not des-Arg9-[Leu8]bradykinin (1 mM) suffused on the cheek pouch significantly attenuated subtilisin (0.1 and 0.5 mM)-induced leaky site formation and an increase in the clearance of FITC-dextran from the cheek pouch (Fig. 1 to 3; each group, \( n = 4 \); \( P < 0.05 \) in comparison to saline (control; open bars).

Effects of an NK1 receptor antagonist on subtilisin-induced responses. Pretreatment with CP-96,345 (5 mg/kg i.v.) had no significant effects on subtilisin (0.1 and 0.5 mM)-induced responses (Fig. 4; each group, \( n = 4 \); \( P > 0.5 \)).

Effects of aprotinin and leupeptin on subtilisin-induced responses. Pretreatment with aprotinin (5 \( \mu \)g/ml) suffused on the cheek pouch had no significant effects on subtilisin (0.1 mM)-induced responses (Fig. 6; each group, \( n = 4 \); \( P > 0.5 \)).

Effects of indomethacin on subtilisin-induced responses. Pretreatment with indomethacin (10 mg/kg, i.v.) had no significant effects on subtilisin (0.1 and 0.5 mM)-induced responses (Fig. 7; each group, \( n = 4 \); \( P > 0.5 \)).

DISCUSSION

There are three new findings of this study. First, we found that subtilisin, a potent serine proteinase elaborated by Bacillus species (13, 17, 29), increases the macromolecular efflux from the in situ hamster cheek pouch. This response is specific because heat-inactivated subtilisin had no significant effects on macromolecular efflux and because aprotinin, a serine proteinase inhibitor, but not leupeptin, a predominantly cysteine proteinase inhibitor, attenuated subtilisin-induced responses.

Second, the edema-forming effects of subtilisin were mediated by the kinin-inhibitory/kinin metabolic pathway and elaboration
of bradykinin, a 9-amino-acid phlogistic mediator (1, 7, 38), through the stimulation of bradykinin B₂ receptors in the cheek pouch microcirculation (28, 30, 38). This conclusion is based on the observations that Hoe 140 and NPC 17647, two structurally distinct selective bradykinin B₂ receptor antagonists, but not des-Arg⁹-[Leu⁸]bradykinin, a selective bradykinin B₁ receptor antagonist, significantly attenuated subtilisin-induced responses (1, 4, 7, 28, 30, 38).

Finally, we found that the intracellular signaling pathway(s) activated by subtilisin in the microcirculation was not related to the stimulation of afferent nerves or the production of prostaglandins because CP-96,345, a non-peptide-selective NK₁ receptor antagonist, and indomethacin, at concentrations previously shown to inhibit capsaicin and substance P-induced increases in macromolecular efflux and cyclooxygenase in the cheek pouch, respectively (8, 24, 27), had no significant effects on subtilisin-induced responses. Collectively, these data indicate that subtilisin increases macromolecular efflux from the in situ hamster cheek pouch in a catalytic site-dependent fashion through local elaboration of bradykinin. This response does not involve the stimulation of local afferent nerves or the production of prostaglandins.

The hamster cheek pouch is an established animal model to study the effects of proinflammatory mediators and environmental toxicants, such as proteinases, bradykinin, and smokeless tobacco, on macromolecular efflux from postcapillary venules in situ (5–10, 14, 20, 24, 26, 28, 33, 38). In this model, solute efflux is determined by two reproducible parameters, leaky site formation and the clearance of FITC-dextran, thereby providing quantitative appraisal of macromolecular transport across postcapillary venules in the cheek pouch during experimental interventions. Importantly, successive infusions of test compounds, such as subtilisin, bradykinin, and smokeless tobacco (7, 28, 38), at appropriate time intervals are associated with reproducible formation of leaky sites and increases in the clearance of FITC-dextran in the absence of tachyphylaxis. Consequently, changes in macromolecular efflux can be tested repeatedly in the same cheek pouch so that each animal serves as its own control. This, in turn, reduces the
overall number of animals required to perform the study and facilitates data analysis.

The above notwithstanding, Giunta et al. (10) showed that there is a delay in the movement of macromolecules into and out of the cheek pouch. This phenomenon is related, in part, to the lack of lymphatic drainage in the cheek pouch which may hamper extension of the observed movement of FITC-dextran to the human oral mucosa. Nonetheless, this anatomic peculiarity enables the investigator to observe changes in the microvascular bed without the need to subtract the possibly confounding effects of lymphatic drainage. The determination that vascular factors, such as subtilisin and bradykinin-like peptides, alter microvascular responses confirms the importance of the hamster cheek pouch mucosa for vascular integrity.

The increase in macromolecular efflux evoked by subtilisin may have been related, in part, to changes in vasomotor tone and/or increase in venular driving pressure in the cheek pouch. However, this possibility seems unlikely because Murray et al. (20) showed that the effects of bradykinin on microvascular transport are dissociated from its effects on microvascular tone in this preparation. Importantly, previous studies using other proinflammatory mediators in the cheek pouch and other microvascular beds have substantiated this notion (16, 34, 36).

The mechanisms underlying bradykinin production during short-term suffusion of subtilisin on the cheek pouch were not elucidated in this study. However, various microbial proteinases, including subtilisin, have been shown to activate the Hageman factor and prekallikrein leading to elaboration of bradykinin (1, 2, 7, 13, 17, 18, 24, 26, 28). For instance, Molla et al. (17) showed that subtilisin derived from Bacillus subtilis activates both the Hageman factor and prekallikrein in the guinea pig skin to produce bradykinin and to elicit plasma exudation. However, Damas et al. (2) showed that plasma exudation triggered by the injection of collagenase into the rat hind paw is mediated through stimulation of local afferent nerves to release substance P. Roxvall et al. (26) showed that indomethacin attenuates trypsin-induced macromolecular efflux from the in situ hamster cheek pouch. Taken together, these data suggest that the mechanisms underlying the edema-forming effects of proteinases in the peripheral microcircula-

![FIG. 6. Effects of subtilisin (0.1 μM) on leaky site formation (black bars, upper panel) and clearance of FITC-dextran (molecular mass, 70 kDa; black bars, lower panel) from the in situ hamster cheek pouch in the absence or presence of leupeptin, a cysteine proteinase inhibitor (10 μg/ml). Values are the means ± the SEM (each group, n = 4 animals). * p < 0.05 in comparison to saline (control; open bars).](http://evl.asm.org/Downloaded from)
tion are species specific, proteinase specific, and microvascular bed specific. Clearly, additional studies using cell and molecular biology techniques are warranted to elucidate the mechanisms underlying bradykinin production during suffusion of subtilisin on the in situ cheek pouch.

The significance of these findings in relation to clinical manifestations of smokeless-tobacco-induced oral mucosa injury and inflammation was not the goal of the present study. Nonetheless, the knowledge of increased mucosal permeability in response to topical exposure to subtilisin is important. Specifically, the accumulation of various smokeless-tobacco-associated chemical carcinogens or viral genetic material from viruses such as the human papillomavirus requires access to the proliferative zone of the oral mucosa to promote malignant changes (12, 15, 19, 23, 28, 34, 37). Subtilisin may facilitate this process by increasing oral mucosa permeability. Further studies are indicated to support or refute this hypothesis.

The above findings notwithstanding, the results of this study indicate a potentially novel mechanism whereby Bacillus species that contaminate smokeless tobacco may injure the oral mucosa of habitual users. We propose that once smokeless tobacco is placed on the oral mucosa Bacillus species sporulate and release subtilisin. This, in turn, activates the kallikrein/kinin metabolic pathway to produce bradykinin, leading to plasma exudation and interstitial edema. This cascade of biologic responses may be amplified by other proteinases released from oral keratinocytes exposed to smokeless tobacco (28). Whether subtilisin activates the kallikrein/kinin metabolic pathway in the oral mucosa directly or stimulates oral keratinocytes, which are the first oral cells exposed to subtilisin, to release bradykinin-forming proteinases remains to be determined.

In summary, we found that subtilisin increases macromolecular efflux from the in situ hamster cheek pouch in a catalytic-site-dependent fashion through local elaboration of bradykinin. This response does not involve the stimulation of local afferent nerves or the production of prostaglandins. We suggest that subtilisin derived from Bacillus species that contaminate smokeless tobacco modulates, in part, the oral mucosa injury and inflammation observed in habitual users.
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