Smokeless tobacco-exposed oral keratinocytes increase macromolecular efflux from the in situ oral mucosa

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Rubinstein, Israel, Xiao-pei Gao, Sergei Pakhlevaniants, and Dolphine Oda. Smokeless tobacco-exposed oral keratinocytes increase macromolecular efflux from the in situ oral mucosa. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R104–R111, 1998.—The purpose of this study was to determine whether supernatants of cultured human oral keratinocytes (HOK) exposed to an aqueous extract of smokeless tobacco (STE) increase macromolecular efflux from the oral mucosa in vivo and, if so, whether bradykinin mediates in part this response. Subconfluent monolayers of HOK were incubated with STE or media, and supernatants were collected 24, 48, and 72 h thereafter. Using intravital microscopy, we found that suffusion of supernatants of STE— but not media-exposed HOK elicited significant concentration- and time-dependent increases in efflux of fluorescein isothiocyanate-labeled dextran (mol mass 70 kDa) from the in situ hamster cheek pouch (P < 0.05). These effects were significantly attenuated by HOE-140 and NPC-17647 but not by des-Arg9,[Leu8]-bradykinin. Proteolytic activity was increased in supernatants of STE but not media-exposed HOK. However, a mixture of leupeptin, bestatin, and DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid had no significant effects on HOK supernatant-induced responses. Collectively, these data suggest that oral keratinocytes modulate smokeless tobacco-induced increase in macromolecular efflux from the in situ oral mucosa in part by elaborating proteases that may account for local bradykinin production. gingiva; inflammation; bradykinin; proteases; hamster

IT IS WELL ESTABLISHED that regular use of smokeless tobacco is associated with oral mucosa injury and inflammation in susceptible individuals (3, 4, 12, 27). One characteristic feature of this process is plasma exudation that elicits interstitial edema and contributes to oral mucosa dysfunction (12).

To this end, Gao et al. (10) showed recently that suffusion of an aqueous extract of smokeless tobacco (STE) onto the in situ hamster cheek pouch elicits a reversible increase in macromolecular efflux from post-capillary venules into the interstitial space. This response was mediated by local production of bradykinin, a potent phlogistic peptide in the oral mucosa (2, 17, 31). However, the mechanisms underlying bradykinin production in the oral mucosa during suffusion of STE were not elucidated in this study.

Previous studies showed that oral keratinocytes, which are exposed directly to smokeless tobacco in the oral mucosa, elaborate proteases, such as trypsin-, elastase-, and collagenase-like enzymes, that have the capacity to produce bradykinin (2, 5, 10, 14, 18, 23, 30). Conceivably, oral keratinocytes could modulate STE-induced increase in macromolecular efflux from the in situ hamster cheek pouch in part by elaborating proteases, which in turn cleave tissue kininogen to produce bradykinin (2).

The purpose of this study was to begin to address this issue by determining whether supernatants of cultured human oral keratinocytes (HOK) exposed to STE increase macromolecular efflux from the in situ oral mucosa and, if so, whether bradykinin mediates in part this response.

METHODS

Preparation of STE

The extract was prepared according to the method of Oh et al. (21) as previously described in our laboratory (8–10, 15, 28). Briefly, 10 g of smokeless tobacco (153 moist snuff; Tobacco and Health Research Institute, University of Kentucky, Lexington, KY) were mixed with 100 ml of serum-free keratinoyte medium (K-SFM; GIBCO BRL, Grand Island, NY) and incubated at 37°C for 2 h. The mixture was then centrifuged at 450 g for 10 min. The supernatant was collected and centrifuged at 13,000 g for 1 h. After adjusting pH to 7.4 with 0.1 N HCl, the resulting supernatant, designated arbitrarily as a:10 aqueous dilution of raw smokeless tobacco (8–11, 13, 15, 16, 28, 32), was filtered through a Millipore filter (pore size, 0.45 µm), divided into 2-ml samples, snap frozen in liquid nitrogen, and stored at −70°C until used.

Cultured HOK

The methods used to culture HOK have been previously described in detail by Oda and colleagues (19, 20, 23). Briefly, specimens of gingiva were removed from healthy individuals undergoing surgery for impacted third molar or were washed immediately with cold sterile phosphate-buffered saline (GIBCO BRL). After removal of excess and damaged epithelium and connective tissue, each specimen was sectioned into small pieces and incubated overnight in 0.4% dispase grade II (Boehringer Mannheim, Indianapolis, IN) at 4°C. The epithelium was then mechanically separated and trypsinized to dissociate epithelial cells into a single cell suspension. After centrifugation, the cells were collected and resuspended in K-SFM. They were seeded on plastic plates and fed every 3 to 4 days with K-SFM. They were seeded on plastic plates and fed every 3 to 4 days. Passage were used in triplicate in these experiments. Cell viability was always >95%, as assessed by morphological examination using phase-contrast microscopy and a 0.1% trypan blue dye exclusion test.

Preparation of Animals

Adult male golden Syrian hamsters weighing 131 ± 1 g (n = 48) were anesthetized with pentobarbital sodium (6...
mg/100 g body wt). A tracheostomy was performed to facilitate spontaneous breathing. A femoral vein was cannulated to inject fluorescein isothiocyanate (FITC)-labeled dextran (mol mass 70 kDa, 40 mg/100 g body wt dissolved in 1.0 ml normal saline and administered over 1 min), the intravascular tracer, and supplemental anesthesia (2-4 mg 100 g body wt -1 h -1). A femoral artery was cannulated to obtain arterial blood samples and to monitor arterial pressure, which did not change significantly during the course of the experiments. Body temperature was kept constant (37-38°C) throughout the experiment using a heating pad.

After these initial procedures, the hamster was transferred to a heated microscope stage. To visualize the microcirculation of the cheek pouch, we used methods previously described in the literature and our laboratory (6-10, 17, 28, 31). Briefly, the left cheek pouch was spread over a small plastic baseplate, and an incision was made in the outer skin to expose the cheek pouch membrane. The avascular connective tissue layer of the membrane was removed, and a plastic chamber was positioned over the baseplate and secured in place by suturing the skin around the upper chamber. The chamber contained the suffusion fluid. This arrangement forms a triple-layered complex: the baseplate, the upper chamber, and the cheek pouch membrane exposed between the two plates. The chamber was connected to a reservoir containing warmed bicarbonate buffer (37–38°C) composed of (in mM) 131.9 NaCl, 2.95 KCl, 1.48 CaCl2, 0.76 MgCl2, and 11.87 NaHCO3. 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 (Sage Instruments, Boston, MA) for administration of supernatants and drugs at a flow rate of 0.2 ml/min into the suffusate. The suffusate flow rate was 2 ml/min.

Determination of Clearance of Macromolecules

The cheek pouch microcirculation was visualized with an Olympus microscope (Jacobs Instruments, Shawnee Mission, KS) 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-labeled dextran. An excitation filter (KP-490) and a heat filter were positioned between the light source and the objective. A barrier filter (510 nm) was positioned between the objective and the beam splitter. Macromolecular leakage was determined by exudation of FITC-labeled dextran, which appeared as fluorescent “spots” or leaky sites around postcapillary venules (6-10, 17, 31). The number of leaky sites was determined by counting three random microscopic fields at predetermined time intervals during each intervention (see Effects of HOK supernatants on macromolecular efflux). The number of leaky sites counted was then averaged and expressed as the number of leaky sites per one microscopic field (0.11 cm2), as previously described in our laboratory (7, 10, 31).

To calculate clearance of FITC-labeled dextran from the cheek pouch, the suffusate was collected at 5-min intervals throughout the experiment by a fraction collector (Cygnus; ISCO, Lincoln, NE). Samples were collected in glass test tubes, and the concentration of FITC-labeled dextran was determined. Arterial blood samples were collected in heparinized capillary tubes (70-µl volume; Scientific Products, McGaw Park, IL) 5 min before injection of FITC-labeled dextran and at predetermined time intervals thereafter during each intervention (see Effects of HOK supernatants on macromolecular efflux). The concentration of FITC-labeled dextran was determined in all plasma samples. To quantitate the concentration of FITC-labeled dextran in the plasma and suffusate, a standard curve for FITC-labeled dextran concentrations versus percent emission was performed on a spectrophotofluorometer (Photon Technology International, Princeton, NJ). The standard, FITC-labeled dextran, was 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-labeled dextran was calculated from the standard curve. In preliminary studies, 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-labeled dextran. Clearance of FITC-labeled dextran was determined by calculating the ratio of suffusate (ng/ml) to plasma (mg/ml) concentration of FITC-labeled dextran and multiplying this ratio by the suffusate flow rate.

Experimental Protocols

Effects of HOK supernatants on macromolecular efflux. The purpose of these studies was to determine whether suffusion of supernatants of STE-exposed HOK increases macromolecular efflux from the cheek pouch. After suffusion of buffer for 30 min (equilibration period), FITC-labeled dextran was injected intravenously and the number of leaky sites and clearance of FITC-labeled dextran were determined for 30 min. Then supernatants of HOK exposed to 1:100, 1:50, or 1:20 aqueous dilution of raw smokeless tobacco or to media for 72 h were suffused for 40 min in an arbitrary order. In another group of animals, culture media to which 1:100, 1:50, or 1:20 aqueous dilution of raw smokeless tobacco, but not HOK, was added was incubated for 72 h and suffused on the cheek pouch for 40 min. The number of leaky sites was determined every minute for 7 min and at 5-min intervals for 140 min thereafter during each intervention (10). Clearance of FITC-labeled dextran was determined before and every 5 min during suffusion of supernatants and for 140 min thereafter. The time interval between subsequent suffusions of supernatant and culture media was =90 min (10). In preliminary studies, we determined that repeated suffusions of supernatants and media for 40 min were associated with reproducible results. In addition, incubation of culture media for 72 h was associated with no visible leaky-site formation or significant increase in clearance of FITC-labeled dextran. The aqueous dilutions of raw smokeless tobacco used in these studies are based on previous reports in the literature (11, 16, 21, 28, 32).

Effects of bradykinin receptor antagonists on HOK supernatant-induced responses. Gao et al. (10) showed that STE-induced increase in macromolecular efflux from the cheek pouch is mediated by local production of bradykinin. The purpose of these studies was to determine whether bradykinin also mediates leaky-site formation and increase in clearance of FITC-labeled dextran from the cheek pouch, elicited by supernatants of HOK exposed to STE. After the equilibration period, FITC-labeled dextran was injected intravenously and the number of leaky sites and clearance of FITC-labeled dextran were determined for 30 min. Then supernatants of HOK exposed to a 1:50 aqueous dilution of raw smokeless tobacco and collected 24, 48, and 72 h thereafter were suffused for 40 min as outlined in Effects of HOK supernatants on macromolecular efflux. Once the number of leaky sites returned to baseline, HOE-140, NPC-17647 (each 1.0 µM), two selective but structurally distinct bradykinin B2 receptor antagonists (2, 10, 26), or des-Arg9,Leu8-bradykinin (1.0 µM), a selective bradykinin B1 receptor antagonist (2, 7,
were suffused for 30 min, and suffusion of supernatants was repeated. The number of leaky sites and clearance of FITC-labeled dextran were determined after each intervention. In preliminary studies, we determined that suffusion of HOE-140, NPC-17647, and des-Arg<sub>9</sub>-[Leu<sub>8</sub>]-bradykinin (each 1.0 µM) for 30 min was not associated with visible leaky-site formation and increase in clearance of FITC-labeled dextran. In addition, suffusion of supernatants for 40 min before and after suffusion of saline (vehicle) for 30 min was associated with reproducible results. The concentrations of HOE-140, NPC-17647, and des-Arg<sub>9</sub>-[Leu<sub>8</sub>]-bradykinin used in these studies are based on previous studies in our laboratory and reports in the literature (2, 7, 10, 26, 31).

Initial characterization of proteases in HOK supernatants. The results of the studies outlined above show that evolution and decay of leaky-site formation and increase in clearance of FITC-labeled dextran elicited by supernatants of STE-exposed HOK is substantially slower than that observed previously during suffusion of exogenous bradykinin on the cheek pouch (2, 6, 17, 31). These data suggest that phlogistic mediators other than bradykinin are elaborated first by STE-exposed HOK and lead to bradykinin production in the cheek pouch. The purpose of these studies was to begin to address this issue by determining whether HOK exposed to STE elaborate proteases capable of producing bradykinin in the cheek pouch.

We used two strategies to test this hypothesis. First, we determined proteolytic activity in supernatants of HOK exposed to a 1:50 aqueous dilution of raw smokeless tobacco or media and collected 24, 48, and 72 h thereafter using a commercially available chromogenic microassay for proteases containing succinylated casein in conjunction with trinitrobenzenesulfonic acid as a substrate according to the manufacturer’s instructions (QuantiCleave protease assay kit II; Pierce Chemical, Rockford, IL). Each 50-µl sample was assayed in duplicate with a blank. Proteolytic activity in media and a 1:50 aqueous dilution of raw smokeless tobacco was also determined. Absorbency at 450 nm was read by a thermoregulated enzyme-linked immunosorbent assay microplate reader (SpectraMAX 340; Molecular Devices, Palo Alto, CA). Proteolytic activity in each sample, expressed as ng/ml after background subtraction, was determined from a standard curve of known concentrations of N-tosyl-L-phenylalanine chloromethyl ketone-trypsin. The sensitivity of this assay is 2 ng/ml.

In another group of animals, we determined whether a mixture of proteinase inhibitors consisting of leupeptin, Bestatin, and β-2-mercaptoethyl-3-guanidinoethyipropionic acid (MGTA), to inhibit serine proteinases, aminopeptidases, and carboxypeptidase N, respectively (9, 31), attenuates the increase in macromolecular efflux from the cheek pouch elicited by supernatants of STE-exposed HOK. After the equilibration period, FITC-labeled dextran was injected intravenously and the number of leaky sites and clearance of FITC-labeled dextran were determined for 30 min. Then supernatants of HOK exposed to a 1:50 aqueous dilution of raw smokeless tobacco and collected 24, 48, and 72 h thereafter were suffused as outlined in Effects of HOK supernatants on macromolecular efflux. Once the number of leaky sites returned to baseline, the mixture of leupeptin, Bestatin, and MGTA (each 10.0 µM) was suffused for 30 min, and suffusion of supernatants was repeated. The number of leaky sites and clearance of FITC-labeled dextran were determined after each intervention. In preliminary studies, we determined that suffusion of the mixture of proteinase inhibitors for 30 min was not associated with visible leaky-site formation or significant increase in clearance of FITC-labeled dextran. The composition and concentration of the mixture of proteinase inhibitors used in these studies are based on previous studies in our laboratory (9, 31).

Data and statistical analyses. When a compound was suffused over the cheek pouch, we determined the maximal change in the number of leaky sites and clearance in FITC-labeled dextran and used it as the response to that compound. Because the number of leaky sites and clearance of FITC-labeled dextran during suffusion of saline (vehicle) returned to baseline between suffusions of compounds, all vehicle 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 < 0.05 was considered significant.

Drugs. FITC-labeled dextran and des-Arg<sub>9</sub>-[Leu<sub>8</sub>]-bradykinin were obtained from Sigma Chemical (St. Louis, MO). Leupeptin and Bestatin were obtained from Peninsula Laboratories (Belmont, CA). MGTA was obtained from Calbiochem (San Diego, CA). NPC-17647 was a gift from Nova Pharmaceutical (Baltimore, MD). HOE-140 was a gift from Hoechst-Roussel Pharmaceuticals (Somerville, NJ). STE and drugs were diluted in saline to the desired concentrations on the days of the experiment.

RESULTS

Effects of HOK Supernatants on Macromolecular Efflux

Suffusion of supernatants of STE-exposed HOK elicited a significant concentration- and time-dependent leaky-site formation and increase in clearance of FITC-labeled dextran from the cheek pouch (Figs. 1–3, each group n = 4, P < 0.05). This response was observed within 30 min of the start of suffusion and was maximal at 45 min. The number of leaky sites and clearance of FITC-labeled dextran returned to baseline 50–75 min after suffusion of supernatants was stopped. Suffusion of saline, media, and supernatants of HOK cultured in media alone for 72 h was not associated with visible leaky-site formation or increase in clearance of FITC-labeled dextran (Fig. 1, each group n = 4, P > 0.5). The number of leaky sites was zero during suffusion of saline and of supernatants of HOK exposed to media alone for 24, 48, and 72 h. Likewise, clearance of FITC-labeled dextran was 15.7 ± 3.1 × 10<sup>−6</sup>, 12.8 ± 2.7 × 10<sup>−6</sup>, 11.9 ± 1.3 × 10<sup>−6</sup>, and 14.2 ± 2.0 × 10<sup>−6</sup> ml/min during suffusion of saline and of supernatants of HOK exposed to media alone for 24, 48, and 72 h, respectively. Suffusion of culture media to which 1:100, 1:50, or 1:20 aqueous dilution of raw smokeless tobacco, but not HOK, was added was incubated for 24, 48, and 72 h and elicited no visible leaky-site formation or significant increase in clearance of FITC-labeled dextran (data not shown; each group n = 4; P > 0.5).

Effects of Bradykinin Receptor Antagonists on HOK Supernatant-Induced Responses

Suffusion of HOE-140 and NPC 17647 (each 1.0 µM) significantly attenuated leaky-site formation and increase in clearance of FITC-labeled dextran elicited by supernatants of HOK exposed to a 1:50 aqueous dilu-
Fig. 1. Effects of supernatants of cultured human oral keratinocytes (HOK) exposed to 1:100, 1:50, or 1:20 aqueous dilution of raw smokeless tobacco (STE) or media for 72 h on leaky-site formation (top) and increase in clearance of fluorescein isothiocyanate (FITC)-labeled dextran (mol mass 70 kDa, bottom) from the hamster cheek pouch. These effects are not related to nonspecific damage to microvascular endothelium, because the number of leaky sites and clearance of FITC-labeled dextran return to baseline once suffusion of FITC-labeled dextran from the cheek pouch elicited by suffusion of supernatants of HOK exposed to a 1:50 aqueous dilution of raw smokeless tobacco for 24, 48, and 72 h (Fig. 3, each group n = 4, P > 0.5).

Initial Characterization of Proteases in HOK Supernatants

Incubation of HOK with a 1:50 aqueous dilution of raw smokeless tobacco was associated with a significant, time-dependent increase in proteolytic activity in the supernatant relative to that of unexposed HOK (Table 1, each group n = 4, P < 0.05). Proteolytic activity was maximal after 72-h incubation of HOK with STE (Table 1). However, suffusion of a mixture of proteinase inhibitors consisting of leupeptin, Bestatin, and MGTA (each 10.0 µM) had no significant effects on leaky-site formation and increase in clearance of FITC-labeled dextran from the cheek pouch elicited by suffusion of supernatants of HOK exposed to a 1:50 aqueous dilution of raw smokeless tobacco for 24, 48, and 72 h (Fig. 3, each group n = 4, P > 0.5).

DISCUSSION

There are several new findings of this study. We found that supernatants of HOK exposed to an aqueous STE elicit significant concentration- and time-dependent increases in macromolecular efflux from the in situ hamster cheek pouch. These effects are not related to nonspecific damage to microvascular endothelium, because the number of leaky sites and clearance of FITC-labeled dextran return to baseline once suffusion of supernatants is stopped. Moreover, suffusion of supernatants of unexposed HOK and of media to which STE, but not cells, was added has no significant effects on macromolecular efflux.

The edemagenic effects of supernatants of STE-exposed cells are mediated in part by local production of bradykinin, because HOE-140 and NPC-17647, two selective but structurally distinct bradykinin B2 recep-
tor antagonists, but not des-Arg9,Leu8\]bradykinin, a selective bradykinin B1 receptor antagonist, significantly attenuate this response. Local production of bradykinin appears to be related in part to proteases elaborated by STE- but not media-exposed HOK, because proteolytic activity in supernatants of the former group increases in a time-dependent fashion. However, a mixture of proteinase inhibitors consisting of leupeptin, Bestatin, and MGTA has no significant effects on supernatant-induced responses. On balance, these data suggest that oral keratinocytes modulate smokeless tobacco-induced increase in macromolecular efflux from the in situ oral mucosa in part by elaborating proteases that may account for local bradykinin production.

The hamster cheek pouch is an established model to investigate the mechanisms mediating the injurious effects of smokeless tobacco in the oral mucosa in situ (1, 8–10, 25, 28). For instance, Suzuki et al. (28) showed that suffusion of an aqueous extract of smokeless tobacco similar to that used in this study attenuates endothelium-dependent vasodilation in the in situ cheek pouch in a specific fashion. Gao et al. (10) showed that stimulation of bradykinin B2 receptors underlies the increase in macromolecular efflux elicited by STE from the cheek pouch (26). The results of the present study extend these observations by showing that supernatants of STE-exposed HOK increase macromolecular efflux from the cheek pouch by stimulating bradykinin B2 receptors, because HOE-140 and NPC-17647 but not des-Arg9,Leu8\]bradykinin significantly attenuate this response.

The increase in macromolecular efflux elicited by STE-exposed HOK supernatants is not related to presence of STE in the culture media, because suffusion of media to which STE but not cells was added and incubated for 24–72 h had no significant effects on supernatant-induced responses. On balance, these data suggest that oral keratinocytes modulate smokeless tobacco-induced increase in macromolecular efflux from the in situ oral mucosa in part by elaborating proteases that may account for local bradykinin production.

The mediators elaborated by STE-exposed HOK appear to be proteases for the following reasons. The evolution and decay of leaky-site formation and increase in clearance of FITC-labeled dextran elicited by STE-exposed HOK supernatants are substantially smaller than those previously reported when STE was suffused directly on the cheek pouch (8–10). In addition, supernatant-induced responses are most likely not related to a time-dependent increase in the number of cells, because Müns et al. (15) showed that STE does not stimulate oral keratinocyte proliferation and because supernatants of HOK exposed to media alone have no significant effects on macromolecular efflux from the cheek pouch. On balance, these data suggest that STE stimulates HOK to elaborate phlogistic mediators in a specific fashion.

The mediators elaborated by STE-exposed HOK appear to be proteases for the following reasons. The evolution and decay of leaky-site formation and increase in clearance of FITC-labeled dextran elicited by STE-exposed HOK supernatants is almost 10-fold.
slower than that observed previously during suffusion of exogenous bradykinin on the cheek pouch (2, 6, 17, 31). They are observed within 30 min of the start of suffusion of STE-exposed HOK supernatants and are maximal at 45 min. Macromolecular efflux returns to baseline 50–75 min after suffusion of supernatants is stopped. By contrast, bradykinin-induced increase in macromolecular efflux from the cheek pouch is evident within 2 min after the start of suffusion and is maximal after 5–7 min. Macromolecular efflux returns to baseline within 30 min after suffusion of bradykinin is stopped (31). Moreover, incubation of HOK with STE for 24–72 h is associated with a significant time-dependent increase in proteolytic activity in the supernatant that parallels the maximal increase in macromolecular efflux from the cheek pouch. Collectively, these data suggest that proteases, not bradykinin, are elaborated by STE-exposed HOK and may account for bradykinin production in the cheek pouch.

Our contention is supported by previous studies showing that oral keratinocytes elaborate proteases known to produce bradykinin (2, 5, 10, 14, 18, 23, 30). The above notwithstanding, a mixture of proteinase inhibitors consisting of leupeptin, Bestatin, and DL-2-mercaptopropionyl glycine (each 10.0 µM). Values are means ± SE, each group n = 4. *P < 0.05 compared with saline (control).

Table 1. Proteolytic activity in supernatants of cultured human oral keratinocytes

<table>
<thead>
<tr>
<th>Condition, µg/ml</th>
<th>Incubation Time, h</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
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<tbody>
<tr>
<td>Media</td>
<td>ND</td>
<td>ND</td>
<td>0.012 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>STE 1:50</td>
<td>ND</td>
<td>0.015 ± 0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOK</td>
<td>0.008 ± 0.002</td>
<td>0.048 ± 0.003</td>
<td>0.043 ± 0.003</td>
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</tr>
<tr>
<td>HOK + STE 1:50</td>
<td>0.034 ± 0.009*</td>
<td>0.525 ± 0.039*†</td>
<td>2.860 ± 0.125*‡</td>
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</tr>
</tbody>
</table>

Values are means ± SE, each group n = 4. STE 1:50, 1:50 aqueous dilution of raw smokeless tobacco; ND, not done; HOK, human oral keratinocytes. *P < 0.05 compared with supernatants of unexposed HOK, †P < 0.05 compared with 48 h, ‡P < 0.05 compared with 24 h. It is not feasible to expose HOK in culture to raw smokeless tobacco because of its cytotoxicity (8–11, 16, 21, and D. Oda, personal communication). Hence, cells are exposed to a diluted aqueous STE. This approach has been previously used by us and other investigators to study the injurious effects of smokeless tobacco in the...
oral mucosa (8–11, 13, 15, 16, 28, 32). To this end, several toxic and carcinogenic constituents of STE have been previously characterized and found to be qualitatively similar to those in raw smokeless tobacco (13). In addition, people place smokeless tobacco onto the oral mucosa, where it is being mixed constantly with saliva, thereby producing, in essence, an aqueous extract adjacent to oral keratinocytes (4, 12, 13). Taken together, these data suggest that the use of an aqueous extract of raw smokeless tobacco to test the hypotheses set forth in the present study is justified.

The constituents of STE that stimulate HOK to elaborate proteases are difficult to characterize because the chemical composition of smokeless tobacco is complex (13, 22). To this end, the role of nicotine, a major constituent of smokeless tobacco, in modulating biologic responses of oral keratinocytes is uncertain (13, 24, 29). Further studies are needed to address this issue.

Perspectives

Oral keratinocytes are exposed directly to smokeless tobacco in the oral mucosa. The interactions between smokeless tobacco and oral keratinocytes to increase macromolecular efflux from the oral mucosa unraveled in this study suggest that these cells could play a role in the pathogenesis of oral mucosa injury elicited by smokeless tobacco.

In summary, we found that oral keratinocytes mediate smokeless tobacco-induced increase in macromolecular efflux from the in situ oral mucosa in part by elaborating proteases that may account for local bradykinin production.

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