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Capsaicin-induced local elevations in collagenase-2 (matrix metalloproteinase-8) levels in human gingival crevice fluid

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Background and objectives: Application of capsaicin on alveolar mucosa provokes pain and neurogenic vasodilatation in the adjacent gingiva. Pain-associated inflammatory reactions may initiate expression of several pro-inflammatory mediators. Collagenase-2 (matrix metalloproteinase-8: MMP-8) is the major destructive protease, especially in the periodontitis-affected gingival crevice fluid (GCF). With this background, we wished to study whether capsaicin stimulation of alveolar mucosa can induce changes in the GCF MMP-8 levels.

Material and methods: For 10 generally and periodontally healthy human volunteers, capsaicin (3%)-moistened filter paper was applied unilaterally to the buccal alveolar mucosa on the anterior maxilla. GCF samples were collected from the tooth at the stimulation site and from several other incisors in the upper jaw. MMP-8 levels and molecular forms in GCF samples were determined by immunofluorometric assay (IFMA) and western immunoblotting, respectively.

Results: Capsaicin stimulation of the alveolar mucosa induced significant local elevations in levels and activation of MMP-8 in GCF of the adjacent teeth. Western immunoblot revealed that both neutrophil- and mesenchymal-type MMP-8 isoforms were elevated and activated, together with 110 kDa high-molecular size MMP-8 species. This capsaicin-evoked MMP-8 elevation lasted several minutes after stimulation. During the experiments, no marked changes occurred in MMP-8 levels in the GCF of distantly located teeth.

Conclusions: These results suggest that capsaicin-evoked neurogenic gingival inflammation can trigger the expression and activation of MMP-8 in GCF of the adjacent teeth.

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Pain and neurogenic flare reactions caused by capsaicin on alveolar mucosa occur in the adjacent gingival tissues (1). This neurogenic response, induced by release of neuropeptides from the peripheral nerve terminals, is thus called axon-reflex-mediated neurogenic inflammation (2). In addition to the neuropeptide release, capsaicin-irritation also provokes expression of several pro-inflammatory mediators, such as interleukin-1β, tumor necrosis factor-α, and prostaglandins (3, 4). Neuropeptides, either directly or through the action of these pro-inflammatory mediators, can induce the secretion of matrix metalloproteinases (MMPs), for example, collagenase-2 (MMP-8) from

immuno-effector cells such as neutrophils, monocytes/macrophages, and fibroblasts, in periodontium (5–8). MMP-8 is known to be not only the major tissue-destructive protease, especially in the periodontitis-affected gingival crevice fluid (GCF) (9–11), but surprisingly also an anti-inflammatory or defensive factor (12, 13). Interestingly, our recent research indicates that painful stimulation of the tooth surrounded by healthy inflammation-free periodontium provokes elevations in GCF MMP-8 levels of the stimulated tooth (14).

Previous studies in rats have shown that the destruction of capsaicin-sensitive nerve fibres prevents the development of neurogenic inflammation in oral mucosa and ligature-induced marginal periodontitis (15). In this investigation, we measured GCF MMP-levels before, during and after the capsaicin irritation of gingival tissue. If capsaicin provokes matrix degrading enzymes in GCF, the study might show further evidence for a functional link between neurogenic inflammation and periodontal tissue destruction in humans. Earlier, we have shown that capsaicin-induced neurogenic flare responses in gingival tissues do not cross the midline of the maxilla (1). Levels of MMP-8 and its molecular forms in GCF were therefore determined bilaterally from the upper incisors during unilateral application of capsaicin on alveolar mucosa of the anterior maxilla.

Material and methods

Subjects

Ten generally and periodontally healthy human volunteers participated. These were healthy graduate students or researchers (six women, four men; mean age 37.5, range 24–45 years), with excellent oral and general health, free from any clinical signs of infection and inflammation in their oral tissues. Informed consent was obtained from each subject before the experiments, according to the ethical guidelines of the Helsinki Declaration of 1975. The Ethics Committee of the Medical Faculty of the University of Helsinki approved the study protocol.

Capsaicin stimulation

Capsaicin (Sigma Chemical Co., St Louis, MO, USA) was dissolved in olive oil to achieve a 3% solution of capsaicin. For stimulation, filter paper (4×1.5 mm) moistened with 10 µl of this solution was positioned in each subject unilaterally on alveolar mucosa at the site of the upper left central incisor (tooth 21) ($n = 10$).

Gingival crevice fluid collection

The GCF samples were collected bilaterally from gingival crevices of several incisors (teeth 22, 21, 11, 12) in the upper jaw as recently described (14). The samples were taken before capsaicin stimulation (pre), during stimulation (stim), and after stimulation (post). Gingival tissue around the teeth near the stimulation site and around the control teeth, situated across the midline of the maxilla, was carefully periodontally and clinically examined. This included measurement of plaque index, gingival bleeding, and probing depths.

None of the volunteers had used antibiotics within the preceding 6 months. In order to avoid blood contamination and possible stimulation of GCF flow during clinical measurements (e.g. probing), GCF samples were collected before any other clinical recordings. The sampling site was isolated with cotton rolls and supragingival plaque was carefully removed. The region was dried with a gentle air stream, and then GCF was collected by using standardized filter paper strips. The strip was placed into the crevice until mild resistance was felt, and left there for 30 s. After that, the strip was immediately placed in to polypropylene tubes. The GCF volume was measured by a standardized weighing method (Mettler AJ 100/GWB) by weighing the polypropylene tube with the standardized filter paper strip inside, before and immediately after the sample collection. This weight difference was used for GCF flow rate (mg/30 s) calculations. Thereafter, each strip was eluted into 300 µl of 20 mM phosphate buffer pH 6.0 containing 0.15 M NaCl and 0.1% Tween 20 (14, 16). The eluates were stored at −70°C prior to analysis.

Western immunoblot and immunofluorometric assay

GCF samples from the stimulation and control sides were studied by western immunoblotting by use of IgG fractions of specific rabbit polyclonal antihuman MMP-8 antibodies and were analysed by quantitated computer image scanning as previously described (6, 9, 14, 16, 17). Human polymorphonuclear leukocytes (PMNs) and rheumatoid synovial fibroblast culture media (6, 9) served as positive controls for PMN- and fibroblast-type MMP-8 isoforms.

MMP-8 concentrations in the GCF samples were determined by a time-resolved immunofluometric assay (IFMA), and the amounts of monoclonal antibodies 8708 and 8706 for MMP-8 were 1.5 µg and 0.5 µg per assay, respectively (6, 10, 16).

Course of the experiments

Each subject sat comfortably in a dental chair. This investigation consisted of two sessions with an interval of one week. In the first session, capsaicin stimulation was performed in 10 human volunteers, for a period of 10 min. Subjective pain levels evoked by capsaicin stimulation were evaluated on a visual analogue scale, VAS (0 = no pain, 100 = the worst imaginable pain intensity). GCF samples were collected from the permanent right and left upper central and lateral incisors (teeth 12, 11, 21, 22). During the experiment, a total of eight samples were taken. Samples one and two were taken before start of capsaicin stimulation, and their average value served as the baseline. Samples three and four (during 5 min) and five and six (during 10 min) were taken during stimulation and their average value served as the stimulation value. Samples seven (post 15 min) and eight (post 30 min) were taken after removal of the capsaicin-containing filter paper. In the second

control session, the GCF samples were similarly collected without capsaicin stimulation from 10 subjects who had participated in the first session. During the experiments, heart rate and mean arterial blood pressure responses were recorded semi-automatically (Omron, Digital blood pressure monitor, HEM-705C, Osaka, Japan) from the left upper arm at the same time as the GCF samples were taken. These heart rate and mean arterial blood pressure measurements were performed in order to clarify whether capsaicin-evoked pain may have induced some systemic stress reactions (18).

Statistical analysis

At each stimulation site, the two pre-capsaicin values (pre 1 and pre 2) of each subject and stimulation site were averaged to a single baseline value for ongoing analysis. Statistical comparisons between baseline and experimental positions were performed by Wilcoxon matched pair tests. A p-value less than 0.05 was considered significant.

Normalizng was done by calculating relative changes as compared to baseline and using these values to compare responses between sites by Wilcoxon matched-pair test.

Results

Evaluation of clinical parameters of sites exposed to capsaicin stimulation and control sites demonstrated that gingival and periodontal health in addition to the general health of the volunteers was excellent. During experiments, GCF flow rate was not affected by capsaicin application. At the capsaicin stimulation site (tooth 21), the average GCF flow rates before, during and after capsaicin were 0.20 ± 0.085 mg/30 s, 0.18 ± 0.08 mg/30 s, and 0.17 ± 0.08 mg/30 s, respectively. The application of capsaicin did not induce visually detectable signs of gingival inflammation. The average pain magnitude estimate on VAS scores was 17 ± 4 ($n = 10$).

Capsaicin stimulation of the alveolar mucosa was effective to induce significant elevations in MMP-8 levels obtained by IFMA measurement in the GCF of the tooth at the stimulation site (= tooth 21) and of the neighbouring tooth (= tooth 22). These elevations in MMP-8 levels already began to appear during stimulation and remained for 30 min after the end of stimulation. No marked changes occurred in MMP-8 levels or in molecular forms of MMP-8 in GCF of the tooth on the contralateral side (= tooth 11). Responses were significantly higher at the capsaicin stimulation side than on the contralateral side (Fig. 1). The control session without capsaicin stimulation showed that repeated measurement itself did not modulate GCF MMP-8 levels (not shown).

Figure 2 shows the representative western immunoblots for molecular forms and degree of activation of MMP-8 in GCF samples at the capsaicin stimulation site (tooth 21, Fig. 2A) and at the contralateral control site (tooth 11, Fig. 2B). Both in the stimulation and control sites, the baseline (pre; lanes 1 and 2) GCF samples contained bands from 60 to 80 kDa, corresponding to PMN-type active- and pro-enzymes, and from 45 to 55 kDa, corresponding to mesenchymal- or fibroblast-type (non-PMN) active- and pro-enzymes. Furthermore, a 110-kDa high molecular-weight-complex MMP-8 species could repeatedly be detected. We found that capsaicin stimulation elevated and activated the high molecular-weight-complex and PMN-type MMP-8 species (lane 4), and non-PMN-type MMP-8 species (lanes 3–6) at the stimulation site (Fig. 2A). Capsaicin stimulation did not induce any marked changes in western blots for MMP-8 of the teeth on the contralateral side (Fig. 2B).

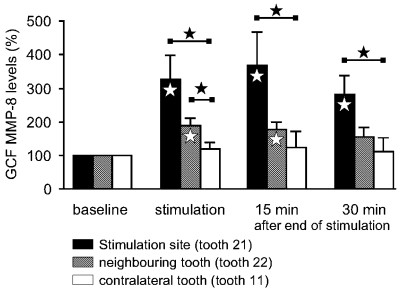

Fig. 1. Relative changes (mean ± SEM, $n = 10$) in matrix metalloproteinase-8 (MMP-8) levels in gingival crevice fluid (GCF) of different teeth during capsaicin stimulation of the alveolar mucosa at the site of the upper left central incisor (tooth 21). Shown are the changes from baseline during capsaicin stimulation, and 15 and 30 min after end of stimulation. The white star denotes significant changes as compared with baseline. The black star marks significant differences between different sites (Wilcoxon matched pair test). Data for the figure is counted from quantitative immunofluorometric assay values normalized to baseline (baseline = 100%).

Capsaicin stimulation, which caused significant elevation in GCF MMP-8 levels at the site of the stimulated tooth, had no marked effects on systemic heart rate or mean arterial blood pressure values (Table 1).

Discussion

In the present study, capsaicin stimulation of the alveolar mucosa evoked significant GCF MMP-8 elevations and activations of the ipsilateral incisors of the anterior maxilla. No significant increases could be detected in MMP-8 levels in GCF contralaterally to the capsaicin stimulation. These results indicate that chemical irritation of gingiva by capsaicin can provoke a local neurogenic inflammation with enhanced proteolytic potential in the closely adjacent gingival tissues, which reaction does not cross the midline in the anterior of the maxilla.

It is well known that capsaicin activates nociceptive C-fibers, inducing a local neurogenic reaction (1, 19) triggered by the release of pro-inflammatory neuropeptides from the nerve terminals (2). In addition to neuropeptides, capsaicin can cause a release of prostaglandin E_2 and proinflammatory cytokines such as IL-1β (3, 4), which alone or in concert can induce the recruited inflammatory and resident cells in periodontium to express MMPs including MMP-8 (8).

In the present study, capsaicin stimulation of the alveolar mucosa caused pain and elevated MMP-8 levels in GCF of the adjacent teeth.

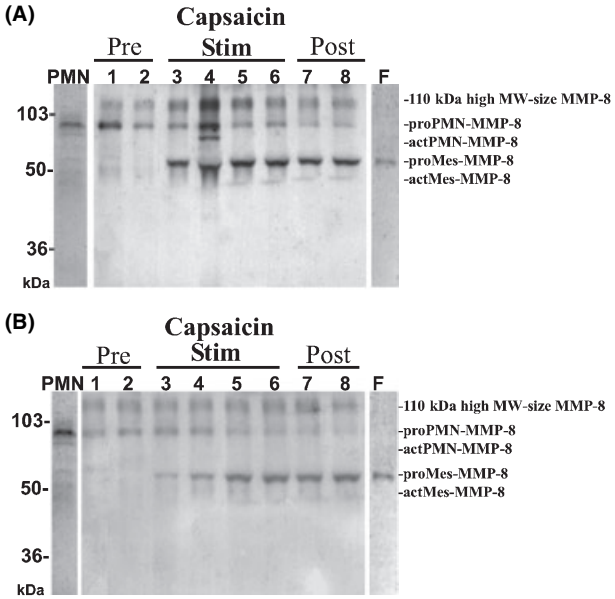

Fig. 2. Representative western immunoblots of molecular forms of matrix metalloproteinase-8 (MMP-8) in gingival crevice fluid (GCF) samples of the tooth (A) at the capsaicin stimulation site (tooth 21), and (B) at the contralateral control site (tooth 11). The GCF samples contained bands at 110 kDa corresponding to high molecular-weight-complex MMP-8 species, at 60–80 kDa corresponding to polymorphonuclear leukocyte (PMN)-type active- and pro-enzymes, and at 45–55 kDa corresponding to mesenchymal-type (non-PMN) active- and pro-enzymes. At the stimulation site (tooth 21), complex and especially PMN-type MMP-8 species (lane 4) and non-PMN-type MMP-8 species (lanes 3–6) were elevated and activated during capsaicin stimulation. PMN indicates PMN-type MMP-8 (lane PMN), and F indicates mesenchymal-type MMP-8 (lane F) from PMN-cultures and rheumatoid synovial fibroblast cultures, respectively. Lanes 1–8 represent molecular forms of MMP-8 in baseline prior to (pre; lanes 1 and 2), during (stim; lanes 3–6), 15 min after (post; lane 7), and 30 min after (post; lane 8) capsaicin stimulation. Mobilities of molecular weight markers appear at the left.

Earlier animal studies have shown that gingival tissues also contain capsaicin-sensitive nociceptive C-fibres (20), the activation of which in these tissues leads to a local axon-reflex-mediated neurogenic inflammation (21, 22). Similarly to capsaicin-induced axon-reflex vasodilatation in the gingiva (1), the present capsaicin-evoked elevations of GCF MMP-8 levels did not cross the midline of the anterior maxilla. These capsaicin-evoked GCF MMP-8 elevations could thus be based on an axon-reflex-mediated neurogenic inflammatory mechanism. These results are in-line with our recent findings showing that experimental stimulation of intrapulpal nociceptive fibers provokes pain and can trigger neurogenic-mediated elevations in GCF MMP-8 levels of the stimulated tooth (14).

The capsaicin stimulation did not affect GCF flow. MMP-8 immunoreactivity increased significantly near the stimulation site during stimulation and for 30 min after the stimulation. Since no signs of gingival inflammation or pocket formation were evident among the subjects, we may assume that the cells within the periodontium were up-regulated to produce MMP-8, and that this elevated expression of MMP-8 seemingly reflects neuropeptide-mediated MMP-8 up-regulation induced by stimulation of nociceptive nerve fibres by capsaicin.

An immunofluorometric assay (IFMA) (6, 10, 23) is a rather sensitive method and determines MMP-8 levels in GCF on a scale of μg/l or ng/ml. The molecular forms of MMP-8 in the GCF from analysed teeth were determined also by western immunoblot method (9, 14, 16, 17). Thus, by two different types of analysis methods utilizing MMP-8 specific monoclonal and polyclonal antibodies (6, 10), we were able to double-check the GCF MMP-8 analysis. Since the individual molecular forms of MMP-8 present in GCF or other oral fluid samples cannot be separately analysed solely by MMP-8 IFMA assay or other available MMP-8 ELISA techniques utilizing anti-MMP-8 antibodies measuring only total levels of MMP-8 immunoreactivity, western immunoblotting was used to accomplish this task (6, 9, 13).

The control experiments showed that the repetitive placing of filter paper strips into the gingival crevice did not raise levels of MMP-8 nor affect its molecular forms in GCF. Thus, we

Table 1. Capsaicin stimulation of the alveolar mucosa elevated local gingival crevice fluid matrix metalloproteinase-8 levels[a] but had no effect on systemic heart rate or mean arterial blood pressure

	Baseline	Capsaicin stimulation	15 min after end of stimulation	30 min after end of stimulation
GCF MMP-8 (μg/l) of the tooth at the stimulation site (tooth 21)	56 ± 20.5	131 ± 39.9*	115 ± 17.3*	98 ± 20.4*
Heart rate (beat/min)	63 ± 3.2	60 ± 2.5	62 ± 2.8	59 ± 2.0
Mean arterial pressure (mmHg)	90 ± 4.4	91 ± 5.5	90 ± 4.7	90 ± 4.0

[a]Quantitative gingival crevice fluid (GCF) matrix metalloproteinase-8 (MMP-8) based on immunofluorometric assay data.
*Significant difference as compared with baseline (Wilcoxon matched pairs). All data is presented as average values (mean ± SEM, $n = 10$) over all subjects.

assume that the MMP-8 elevation together with its molecular forms during capsaicin stimulation was not caused by the measurement method itself, but instead was due to an active response triggered by the application of capsaicin on the alveolar mucosa.

The biological roles of MMP-8 *in vivo* are currently not completely clarified (12, 13, 24, 25). Based on its established catalytic proteolytic competence, MMP-8 is considered to be involved in tissue remodelling and destruction during inflammation (10, 11, 24, 25). In this respect, MMP-8 has been regarded as among the key and pivotal tissue-destructive proteinases associated with progression of periodontal diseases (9, 10, 23).

Polymorphonuclear leukocytes (PMNs) are regarded as the major source of 60- to 80-kDa MMP-8 isoforms in humans (11, 25), but the 45- to 55-kDa non-PMN-8 isoform MMP-8 has also been reported to be expressed by gingival fibroblasts, epithelial cells, endothelial cells, and activated monocytes/macrophages (9, 24, 26). In addition, Owen *et al.* (13) recently offered evidence that PMNs are furthermore associated with catalytically competent membrane-bound MMP-8 with molecular size ranging from 30 to 110 kDa. Furthermore, Balbin *et al.* (12) and Owen *et al.* (13) described that MMP-8, in addition its surrogate mediator of tissue destruction, also exerts unexpected new anti-inflammatory and defensive roles, especially in the lung during LPS-mediated inflammation. It is worth of note, that the detected molecular forms (65–110 kDa) of PMN-type MMP-8 in GCF may represent both released soluble or membrane-bound forms of PMN-type MMP-8 (13, 27, 28). Thus, in the capsaicin-stimulated GCF samples, the 110-kDa high-molecular-weight MMP-8 species may, at least partially, represent membrane-bound MMP-8 species shed or escaped from PMNs. Nonetheless, all molecular species of MMP-8, soluble or shed membrane-bound, were elevated, and both the PMN- and non-PMN-type MMP-8 isoforms were also partially activated by capsaicin stimulation.

Stress has been shown to modulate some periodontitis-relevant immune-parameters in GCF (29). Since the present changes in GCF MMP-8 levels were restricted only to the ipsilateral side in relation to capsaicin stimulation, it seems improbable that the stress mechanism had an effect on these results. Moreover, the current findings that blood pressure and heart rate were not markedly modulated by the painful capsaicin stimulation do not favor stress mechanisms underlying the present MMP-8 elevations, either.

In summary, the present investigation demonstrates that chemical irritation of gingivomucosal tissues by capsaicin produces local elevations of levels of a potent host-tissue destructive protease, MMP-8, in GCF of the neighbouring teeth. Similarly to capsaicin-induced neurogenic vasodilatation in gingiva (1), the capsaicin-evoked response in GCF of MMP-8 does not cross the midline of the anterior maxilla. It is likely that capsaicin-evoked GCF MMP-8 elevation is based on an axon-reflex-mediated neurogenic inflammatory reaction in human gingivomucosal tissues. It is possible that, similar to the experimentally induced neurogenic inflammation, bacterial toxins and metabolites elicited clinical gingival inflammatory reactions do not spread from one side to the other of the anterior maxilla.

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