Increased Leukotriene Concentration in Submandibular Glands from Rats with Experimental Periodontitis

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Abstract

Mucin release by submandibular gland is increased in rats with ligature induced-periodontitis and the increment is associated with activation of sympathetic nervous system. Besides, isoproterenol EC_{50} and maximal effect are altered in rats with ligature. Here we studied the relation between the inflammatory mediators with mucin release by submandibular gland in a rat model of experimental periodontitis. Hydrocortisone, sc, abolished the basal increment of mucin. NO and PGE_2 were not increased in submandibular glands and neither, the inhibition of NO synthesis nor the inhibition of PGE_2 synthesis, could modify the changes induced by periodontitis. Cysteinyl-leukotrienes were significantly increased and the increment was abolished by 5×10^{-6} M NDGA. In presence of NDGA, basal mucin secretion and isoproterenol concentration response curve, in rats with ligature, were similar to controls. The cysteinyl-leukotriene receptor antagonist, FPL 55712, inhibited the increment of non-stimulated mucin in rats with ligature. This results point to cysteinyl-leukotrienes as the mediators involved in the increase mucin secretion observed in rats with experimental periodontitis. We can conclude that the cytokines liberated during the periodontal disease induced the synthesis and liberation of leukotrienes in the submandibular gland which in turn induce mucin release through the activation of the sympathetic fibers.

Introduction

Periodontitis is a destructive inflammatory disease of the supporting tissues of the teeth. This disease is primarily related to chronic plaque accumulation. Putative periodontopathic bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia* or *Actinobacillus actinomycemcomitans* are suspected to play a role in the periodontal disease process. These bacteria produce a large number of proteolytic enzymes, the majority being cysteine proteinases, which degrade plasma proteins, inmmunoglobulins, collagen, and fibronectin (1). Furthermore, these pathogenic bacteria provoke an

immune response that results in the release of cytokines which trigger PMNs, macrophages and fibroblasts which in turn release a range of inflammatory mediators, including arachidonic acid metabolites such as prostaglandins and leukotrienes (2), and nitric oxide (3).

Patients with periodontal disease have differences in the protein composition of whole saliva. It was reported that the total protein concentration was higher in whole saliva from periodontitis subjects (4). Whole saliva is a mixture of gingival fluid and the secretions of the major and minor salivary glands. The composition of saliva reflects the nature and amplitude of the host response to a periodontal microbial challenge (5). In general, the major factors affecting the protein concentration and composition of whole saliva are the salivary flow rate, the protein concentration of the contributing glandular saliva and the contribution of crevicular fluid protein. The enhanced protein concentration in periodontitis subjects cannot be attributed to a changed flow rate since any statistically significant difference in mean saliva flow rates was found (6, 7). Thus, the elevated protein levels are most likely due to enhanced synthesis and secretion by the individual glandular saliva. Cystatin C as well as amylase is present in almost two-fold higher concentrations in parotid saliva of periodontitis patients (8). Salivary secretory immunoglobulin A (IgA) is considered as an important first line of defense mechanism in the oral cavity. In healthy subjects with regular hygiene the development of plaque-induced gingivitis is associated with increased salivary gland output and increased total IgA output levels in stimulated parotid saliva (9). In addition, it was reported higher concentrations of epidermal growth factor in glandular saliva from juvenile periodontitis subjects (10). The mechanism underlying glandular output has not been established experimentally. However, a large number of animal studies have provided evidence that expression of specific salivary proteins in rodents is influenced by various external stimuli. Rats treated with β_1-adrenergic agonists, e.g. isoproterenol, produced dramatically more of submandibular rat cystatin S and, at the same time, less kalllicrein (11). Comparable effects on glandular production of cystatin S were found in rats as a consequence of irritating actions such as incisor amputations (12). Recently, we have studied mucin release by submandibular gland in a model of periodontal disease in rats. We found significantly increased mucin secretion in rats with periodontal disease and this increment was abolished in the presence of β_1-adrenergic antagonists. Furthermore, it was observed a desensitation to isoproterenol-stimulated mucin release

and a decrease in isoproterenol maximal effect. These results strongly suggested the activation of the sympathetic nervous system in response to the inflammatory disease (13).

The sympathetic nervous system plays a central role in establishing communication between the central nervous system and the immune system during inflammation. Inflammatory states induce the simultaneous expression of inflammatory mediators and the activation of the sympathetic nervous system. Increasing evidence suggests a reciprocal interaction between these two responses. Catecholamines released from the sympathetic nervous system can alter the production of pro-and anti-inflammatory mediators, and accordingly influence disease activity or clinical symptoms in a variety of pathophysiological states (14). Catecholamine release generally occurs in response to stressful conditions caused by cognitive stimuli or non-cognitive stimuli, such as the immune/inflammatory response itself (15).

The good evidence that the increase of mucin release by submandibular glands from rats with experimental periodontitis was due to an activation of sympathetic nervous system (13), and the association of this system with the immune/inflammatory response, let us to conduct this study to evaluate the association between inflammatory mediators and mucin release in rats with a model of periodontal disease. We also attempted to throw light on the signaling pathway involved in the activation of sympathetic nervous system.

Materials and Methods

Animals

Male Wistar rats weighing 250–300 g were lightly anaesthetized with a mixture of Ketamine and Xilazine (50 and 5 mg/ k respectively). A black thread was placed around the cervix of the two lower first molars and knotted mesially (13). Experiments were carried out 22 days after the rats were subjected to ligature-induced periodontitis.

Animals had free access to food and water until the night before experiments when food, but not water was withdrawn. Animal care was provided according to “The Guide to the Care and Use of Experimental Animals” (DHEW Publication, NIH 80-23).

Measurement of mucin release

Extirpated submandibular glands were detached from free connective tissue and sliced into pieces approximately 2–3 mm thick and 15 mg wet weight with a razor blade. Gland slices were incubated for 30 min in 500 μl of Krebs Ringer bicarbonate medium (KRB), pH 7.4 bubbled with 5% CO_2 in O_2 at 37° C. When used, inhibitors (propranolol, atenolol and FPL 55712 from Sigma Chemical Co., St Louis, MO, USA), were included from the beginning of the incubation and isoproterenol (Sigma Chemical Co., St Louis, MO, USA) was added in the last 15 min of the incubation time. Placing the tubes on ice then stopped the reaction. Glands were homogenized in 50 mM sodium acetate buffer, 25 mM Cl_2Mg, pH 5.8, supplemented with protease inhibitors (0.1 mM phenylmethyl-sulfonyl fluoride, 1 mM sodium ethylenediaminetetra-acetate and 1 mM iodoaceamide) at 4° C and centrifuged at 900 × g for 15 min. Mucin was determined in the supernatants (residual mucin content in the gland) and in the incubation medium (mucin released) using the Alcian Blue method described by Hall et al. (16) and modified by Sarosiek et al. (17). Briefly, aliquots of diluted supernatants (1:100) or medium (1:10) were incubated for 30 min in a 1 % solution of Alcian Blue in 50 mM sodium acetate buffer 25 mM Cl_2Mg, pH 5.8 under constant agitation at room temperature. Following incubation, the samples were centrifuged for 20 min at 3000 rpm pellets washed in 95% ethanol, vortexed gently for 10 s and after 5 min, centrifuged for 20 min at 3000 rpm. Mucin–dye complexes were dissociated by the addition of a 1:2 dilution of Aerosol OT (Sigma Chemical Co., St Louis, MO, USA) in distilled water, brief mixing and sonication. Subsequently, samples were extracted with

equal volumes of ethyl ether under vigorous shaking. The resulting solution was centrifuged for 15 min at 3000 rpm and the dye concentration determined spectrophotometrically at 605 nm in the aqueous layer. Mucin released is expressed as percentage released from total (residual + release) mucin content in the gland (% of total) (18).

***In vivo* experiments**

Rats received vehicle (polyethylene glycol) or hydrocortisone 1 mg/ k (Sigma Chemical Co., St Louis, MO, USA), subcutaneously (sc), daily treatment for 3 days before the experiments, and the measurement of mucin release was performed as stated above.

Nitric Oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in submandibular glands using L-[U-^{14}C]arginine as substrate as described earlier (19). Briefly, 50-60 mg of submandibular slices were incubated with 0.4 μCi L-[U-^{14}C] arginine (Amersham Pharmacia Biotech, Buckinghamshire, England, about 300 mCi/ mmol) in 500 μl of KRB solution pH 7.4 gassed with 5 % CO_2 in O_2 for 30 min at 37º C. After incubation the tissue was homogenized, centrifuged at 10,000 g for 10 min and [^{14}C]citrulline in the supernatants was separated by ion exchange chromatography on AG 50 W resin (Biorad). When used the NOS inhibitor N^G-Monomethyl-arginine (L-NMMA) (Sigma Chemical Co., St Louis, MO, USA), was included from the beginning of the incubation time. Nitric oxide production (measured as pmol of [^{14}C]citrulline) was expressed as picomol of [^{14}C]citrulline/ g of tissue wet weight (pmol/g wet wt).

Determination of prostaglandin production

Submandibular slices (55 mg) were incubated in 160 μl of KRB solution, pH 7.4, gassed with 5 % CO_2 in O_2 for 30 min at 37º C. When used, the cyclooxygenase (COX) inhibitors indomethacin (Sigma Chemical Co., St Louis, MO, USA) and 5-Bromo-2(-4 fluorophenyl)-3 [4-(methylsulfonyl) phenyl] thiphene (DuP 697) (Tocris Cookson Inc. Ellisville MO, USA) were included from the beginning of the incubation time. After homogenization all procedures employed were those indicated in the protocol of

Prostaglandin E_2 Biotrak Enzyme Immuno Assay (ELISA) System (Amersham Biosciences, Piscataway, NJ, USA). The results are expressed as picogram of PGE_2/ milligram of tissue wet weight (pg/ mg wet wt).

Measurement of leukotriene concentration

Submandibular glands were incubated in 400 µl of KRB solution, pH 7.4, gassed with 5 % CO_2 in O_2 for 30 min at 37º C. When used, nordihydroguaiaretic acid (NDGA) (Sigma Chemical Co., St Louis, MO, USA) was included from the beginning of the incubation time. After homogenization all procedures employed were those indicated in the protocol of Enzyme Immuno Assay kits Cayman Chemicals (Ann Arbor MI).The results are expressed as picogram of cysteinyl leukotrienes/ milligram of tissue wet weight (pg/ mg wet weight)

Statistical analysis

Statistical significance of differences was determined by the Newman-Keuls test after analysis of variance. Differences between means were considered significant at $P< 0.05$. Fitting dose–response curves were done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA, USA).

Results

First we investigated the relation between inflammatory mediators and mucin release by studying the effect of hydrocortisone administration on mucin secretion by submandibular glands from rats with periodontal disease. Accordingly with our previous results (13) we observed an increment of basal mucin secretion in rats with ligature. The administration of 1 mg/ k of hydrocortisone sc during 3 days prevented the increase of mucin release by submandibular glands from rats with periodontal disease,

reaching values similar to controls (Figure 1, A). This result was compared with those obtained with the β-adrenoceptors antagonists, propranolol and atenolol, both in concentrations of 5 x 10^{-7} M. As observed previously (13), the increment of basal mucin secretion in submandibular glands of rats with ligature was inhibited by propranolol and atenolol (Figure 1, A).

Figure 2 A shows the total NOS activity in submandibular glands from control and with experimental periodontitis rats. As can be seen the values of NOS activity did not change in rats with periodontitis and the non-selective NOS inhibitor L-NMMA, 5 x 10^{-6} M significantly inhibited NOS production in both groups. This result indicates that in non-stimulating conditions total NOS activity is not modified by periodontitis. Figure 2 B shows the isoproterenol concentration-response curve on mucin secretion. As observed previously (13), in rats with periodontitis there was an increase of isoproterenol EC_{50} values (7.74 x 10^{-9} M and 8.97 x 10^{-8} M for control and with ligature rats respectively) with a decrease of isoproterenol maximal effect (14.23 ± 0.46 and 11.8 ± 0.47 for control and with ligature rats respectively). In order to see whether NOS participated in basal and isoproterenol-stimulated mucin secretion by submandibular glands from control and with experimental periodontitis rats, we carried out the concentration-response assays in the absence and the presence of 5 x 10^{-6} M L-NMMA. Figure 2 B shows that the inhibition of NOS activity by L-NMMA did not modify neither basal nor isoproterenol-induced mucin secretion in both groups.

To investigate whether other pro-inflammatory mediator could be implicated in the mucin release alteration by submandibular glands from rats with experimental periodontitis, we evaluated PGE_2 production in submandibular glands from control and with experimental periodontitis rats. Since PGE_2 synthesis in inflammation is achieved through the inducible COX-2 activity, we evaluated its production in the absence and the presence of the non-selective COX inhibitor indomethacin (5 x 10^{-6} M) and the selective COX-2 (inducible) inhibitor, DuP 697 (10^{-8} M). Figure 3 A shows that PGE_2 production was similar in both groups, control and with ligature, and that only the non-selective COX inhibitor, indomethacin, had inhibitory effect, indicating the absence of COX-2 activity. With the aim to evaluate the participation of PGE_2 in basal and isoproterenol-induced mucin secretion in submandibular glands from rats with experimental periodontitis and control, we carried out the concentration-response assays in the absence and the presence of 5 x 10^{-6} M indomethacin. Figure 3 B shows that the

differential basal and isoproterenol-induced mucin secretion in control and in rats with ligature was not alter in the presence of the COX inhibitor, indomethacin,.

Leukotrienes are potent lipid-derived inflammatory mediators derived from lipoxygenation of arachidonic acid. Further metabolism leads to the formation of either lekotriene B_4 (LTB_4) or cisteinyl-leukotrienes. These lipoxygenase products had been detected in crevicular fluid from subjects with periodontal disease (20). Thus, we evaluated its concentration in submandibular glands from rats with ligature and control. Figure 4 A shows the concentration of cisteinyl-leukotrienes in the absence and presence of the lipoxygenase inhibitor NDGA at concentrations of 5×10^{-6} M, in submandibular glands from rats with ligature and control. As can be seen, in submandibular glands from rats with ligature cisteynil leukotrienes were significantly increased and the increment was abolished in the presence of NDGA. In order to asses whether lipoxygenase products were involved in basal and isoproterenol-induced mucin secretion in rats with ligature and control, we carried out the concentration-response assays in the absence and the presence of 5×10^{-6} M of NDGA. Figure 4 B shows that, in the presence of NDGA, basal and isoproterenol-induced mucin secretion by submandibular glands from rats with experimental periodontitis were similar to control. These results clearly suggest that lipoxygenase products are responsible of the increased basal mucin secretion and the altered isoproterenol effect observed in submandibular glands from rats with experimental periodontitis. On the other hand, NDGA did not modify neither basal nor isoproterenol concentration-response curve of control rats (Figure 4 B).

In order to evaluate whether cysteinyl-leukotrines were acting through their own receptor we studied the effect of the cysteinyl leukotriene receptor antagonist, FPL 55712, on basal mucin release by submandibular glands from rats with periodontitis and controls. Table 1 show that in the presence of FPL 55712 basal mucin release from rats with periodontititis was similar to that of controls.

Experimental periodontitis induced a significant increase in submandibular gland weight and a depletion of residual mucin concentration in non-stimulated glands (Table 2). The submandibular glands weight increased 15 % in rats with ligature and the depletion of mucin was of 25 %. In the presence of 5×10^{-6} NDGA, mucin concentration of glands from periodontitis rats increased, reaching values similar to controls (Table 2). On the other hand, NDGA did not modify the increase gland weight

in rats with ligature. This fact could explain why the maximal effect of isoproterenol in submandibular glands from rats with ligature, in the presence of NDGA, was similar to that of controls (Figure 4 B).

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Discussion

Periodontal disease triggers an immune/ inflammatory response that results in the release of cytokines which triggers PMNs, macrophages and fibroblasts which in turn release inflammatory mediators (21). This disease is accompanied by changes in the protein composition of saliva. Enhanced levels of cystatins, IgA and epidermal growth factor have been described (8). Our previous results showed an increase of mucin release from submandibular gland in a rat model of periodontal disease associate with a sympathetic hyperactivity (13). In this paper we studied the association of

immune/ inflammatory mediators with the changes observed in mucin release by submandibular glands from rats with experimental periodontitis.

Our finding show that the administration of hydrocortisone during 3 days prevented the increase of non-stimulated mucin release in rats with periodontitis as also did the β-adrenergic blockers. These results strongly support the view that the increment of mucin release in submandibular glands from rats with experimental periodontitis is associated with a neuroimmune/ inflammatory response.

It is well known that hydrocortisone inhibits cytokines release from inflammatory cells (22) such as tumor necrosis factor alpha (TNF-α), interleukin -1β (IL-1β) and interferon-γ (IFN-γ) and that these cytokines can generate large amount of nitric oxide (NO) following bacterial infection (23). On the other hand, hydrocortisone inhibits phospholipase A_2, resulting in an inhibition of prostaglandins and leukotrienes production (24).

Enhanced production of NO has been demonstrated in periodontal disease (25) and gingival tissues from patients with chronic periodontitis have higher levels of iNOS protein and mRNA than healthy tissue (26). On the other hand, NO is involved in blood flow and nerve-evoked protein output in submandibular glands (27). Thus, we decided to investigate NO production and its association with mucin release in submandibular glands from rats with ligature and control. Our results showed that submandibular glands from rats with periodontitis and control generated the same amount of NO that was inhibited by the non selective NOS inhibitor L-NMMA. This result indicates that, although NO is an inflammatory mediator in salivary gland disease (28) 22 days of ligature do not induce changes in NO production in subamndibular gland. As expected, basal mucin release and isoproterenol concentration-response curves were not modified in the presence of the NOS inhibitor L-NMMA indicating that NO was not the periodontal inflammatory mediator involved in the increase mucin secretion observed in submandibular glands from rats with ligature. This result confirms our previous report (13)

Because NO was not found as the mediator involved in mucin secretion in submandibular gland from rats with periodontal disease, we investigated the participation of PGE_2, which is other inflammatory mediator present in crevicular fluid from patients with periodontal disease (29) and its production is inhibited by hydrocortisone (24). PGE_2 modulates the secretory activity of rat salivary glands (30)

and also, PGE_2 content, catalyzed by the inducible isoform COX-2 (31), is increased in salivary glands inflammatory diseases (32). Thus, we studied PGE_2 production by submandibular gland from rats with periodontal disease in the presence of indomethacin as COX-inhibitor and the selective COX-2 inhibitor DuP 697. We ruled out the participation of PGE_2 as the inflammatory mediator involved in mucin release in rats with periodontitis because PGE_2 concentration was similar in submandibular gland from control and with ligature rats and COX-2 did not seem to participate in PGE_2 production in both groups. Moreover, while indomethacin decreased PGE_2 release, the COX-inhibitor did not modify neither basal nor isoproterenol-induced mucin secretion. This result agrees with the *in vivo* effect of indomethacin (13)

Leukotrienes are potent lipid-derived inflammatory mediators derived from lipoxygenation of arachidonic acid. Leukotriene B_4 (LTB_4) and cysteinyl-leukotrienes has been detected in gingival crevicular fluid from periodontitis patients (20). In the human airway epithelium, leukotriene receptor system is one of the mechanisms related to *MUC2/5AC* gene expression and mucin secretion (33). In rat salivary glands leukotriene C_4 and D_4 modulate methacholine and substance P-induced salivary secretion (34). Leukotriene production is also inhibited by hydrocortisone (24). Thus, we investigated its concentration and its participation on mucin release in submandibular gland from rats with periodontitis and control. The concentration of cysteinyl-leukotrienes was increased in submandibular gland from rats with periodontal disease and the increment was inhibited by a lipoxygenase inhibitor. Moreover, isoproterenol-induced mucin secretion in submandibular gland from rats with ligature, in the presence of NDGA, was similar to that of control. These results strongly point leukotrienes out to be the mediators of inflammation involved in sympathetic-mucin release during periodontal disease. There are previous studies in animal model supporting the role of leukotrienes in host defense against bacterial infections (35), and in addition, it was shown that administration of LTB_4 to mice enhanced bacterial clearance (36). Another mechanism triggered by LTs is the release of defensins (37) a group of small antimicrobial peptides of the innate immune system. In human, β-defensins are expressed in the oral mucosa, gingival tissue, tongue, salivary glands, and they appear in saliva (38). Here, we found that lipoxygenase products induce an increase of mucin secretion by submandibular gland from rats with periodontitis. Mucin play a major role in the oral defense mechanism by maintaining the viscoelastic

properties of saliva, participating in the formation of protective oral mucosal mucus coat and tooth enamel pellicle and promoting bacterial aggregation and clearance from the oral cavity (39).

Regarding the above observations we can hypothesize that, in our experimental conditions, submandibular gland from rats with 22 days of ligature were not affected by an inflammatory process which involved NO and PGE_2, because both mediators of inflammation were not increased. But cysteinyl leukotrienes, which play an important role in defensive mechanisms, were increased. Experimental model of periodontitis in the rhesus monkey revealed a sequence of inflammatory mediators in the crevicular fluid that was related with changes in the clinical disease expression. Leukotrienes showed an early rise at 1 month, while PGE_2 had higher values at 2 to 3 months (21). It is possible that cytokines, released from the periodontal inflammatory process, reach the submandibular gland and activate the local immune cells to produce leukotrienes, being this fact perhaps, the first manifestation of the involvement of submandibular gland in the periodontal disease. The participation of immune cells from the own gland is suggested by the inhibition observed with the lipoxygenase inhibitor, NDGA, when was included in the incubation medium. The overwhelming evidence suggests that leukotriene synthesis and subsequent release in experimental animals and in human are associated with certain pathophysiological events, such as asthma, adult respiratory disease syndrome, septic shock, psoriasis, inflammatory bowel disease and myocardial ischemia. Recent studies have explored the participation of leukotrienes in the fine tuning of the immune system and, evidence suggests a neuromodulatory role of leukotrienes and other lipoxygenase metabolites in the central nervous system (40).

Other finding from our experiments was that cysteinyl-leukotrienes are acting through their own receptor because their action was inhibited with the cysteinyl leukotriene receptor antagonist, FPL 55712. It is known that in human airway epithelial cells the leukotriene receptor system is one of the mechanisms related to mucin gene expression and secretion (33). But, in our study, the increase in mucin release in glands from periodontitis rats, triggered by leukotrienes, is achieved through sympathetic activation, suggesting that mucin secretion is an indirect effect. It was established that certain tissue may contain elements with the ability to release mediators when exposed to LTB_4 (41). It is likely, that in the submandibular gland, the presence of lipoxygenase products provoke the release of sympathetic mediators. At least 30 different

metabotropic and four different ionotropic receptors have been found to control the amount of transmitter being released from a sympathetic axon terminal (42) and, a modulation of sympathetic neural system by leukotrienes has been described in rat aorta (43). Thus, we can speculate that leukotrienes induce noradrenlaine release through a facilitatory presynaptic effect resulting in an increase of mucin release. The interaction between the sympathetic nervous system and the immune system during inflammation has been described, and one aspect of the communication is that the release of transmitters from the sympathetic varicosities may be subject to modulation through various receptors located pre-synaptically (14). Furthermore, immune cells express various adrenergic receptors and immune/ inflammatory process is regulated upon occupation of these cell surface structures (14). Then, it is tempting to speculate that, in submandibular gland from rats with periodontitis, leukotrienes activate sympathetic neural system which in turn induces mucin release and activates immune cells to release leukotrienes, generating a feed-back regulatory mechanism of the immune response.

In Figure 5 we summarize the hypothetic events that provoke an increase in mucin release in submandibular gland from rats with experimental periodontitis.

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References

1. Mayrand D, Holt SC. Biology of asaccharolytic black-pigmented bacteroides species. *Microbiol Rev* 1988; **52**: 134-152.
2. Offenbacher S, Odle BM, Van Dyke TE. The use of crevicular fluid prostaglandins E_2 levels as a predictor of periodontal attachment loss. *J Periodontal Res* 1986; **21**: 101-112.

3. Lohinai Z, Stachlewitz R, Virag L, Székely AD, Hasko G, Szabó C. Evidence for reactive nitrogen species formation in the gingivomucosal tissue. *J Dent Res* 2001; **80**: 470-475.
4. Henskens YMC, Vand der Velden U, Veerman ECI, Nieuw Amerongen AV. Protein, albumin and cystatin concentrations in saliva of healthy subjects and of patients with gingivitis or periodontitis. *J Periodontal Res* 1993; **28**: 43-48.
5. Mashayekhi F, Aghahoseini F, Rezaie A, Zamani MJ, Khorasani R, Abdollahi M. Alteration of cyclic nucleotides levels and oxidative stress in saliva of human subjects with periodontitis. *J Contemp Dent Pract* 2005; 6: 46-53.
6. Aguirre A. Testa-Weintraub LA, Banderas JA, Dunford R, Levine MJ. Levels of salivary cystatins in periodontally healthy and diseased older adults. *Archs Oral Biol* 1992; **37**: 355-361.
7. Shern RJ, Fox PC, Li SH. Influence of age on secretory rates of the human minor salivary glands and whole saliva. *Archs Oral Biol* 1993; **38**: 755-761.
8. Henskens YMC, Van den Keijbus PAM, Veerman ECI, Van der Weijden GA, Timmerman MF, Snoek CM, Van der Velden U, Nieuw Amerongen AV. Protein composition of whole and parotid saliva in healthy and periodontitis subjects. Determination of cystatins, albumin, amylase and IgA. *J Periodont Res* 1996; **31**: 57-65.
9. Seemann R, Hägewald SJ, Sztankay V, Drews J, Bizhang M, Kage A. Levels of parotid and submandibular/sublingual salivary immunoglobulin A in response to experimental gingivitis in humans. *Clin Oral Invest* 2004; **8**: 233-237.
10. Hormia M, Thesleff L, Perheentupa J, Pesonen K, Saxén L. Increased rate of salivary epidermal growth factor secretion in patients with juvenile periodontitis. *Scand J Dent Res* 1993; **101**: 138-144.
11. Bedi GS. The effect of adrenergic agonists and antagonists on the expression of proteins in rat submandibular and parotid glands *Crit Rev Oral Biol Med* 1993; **4**: 565-571.
12. Yagil C, Barka T. Induction of a specific (LM) protein in the submandibular gland of the rat by repeated amputation of the lower incisor teeth. *Am J Anat* 1986; **177**: 513-518.

13. Busch L, Sterin Borda L, Borda E. β-adrenoceptor alterations coupled with secretory response and experimental periodontitis, in rat submandibular glands. *Archs Oral Biol* 2008; **53**: 509-516.
14. Haskó G. Receptor-mediated interaction between the sympathetic nervous system and immune system in inflammation. *Neurochem Res* 2001; **26**: 1039-1044.
15. Besedovsky HO, del Rey A. Immune-neuro-endocrine interactions: Facts and hypotheses. *Endocr Rev* 1996; **17**: 64-102.
16. Hall RL, Miller AC, Peatfield PS, Richardson I, Williams I, Lampert L. A colorimetric assay for mucus glycoprotein using Alcian blue. *Biochem Soc Trans* 1980; **8**: 72-73.
17. Sarosiek J, Rourk RM, Piascik R, Namiot Z, Hetzel DP, McCallum RW. The effect of esophageal mechanical and chemical stimuli on salivary mucin secretion in healthy individuals. *Amer J Med Sci* 1994; **308**: 23-31.
18. Busch L, Borda E. Signaling pathways involved in pilocarpine-induced mucin secretion in rat submandibular glands. *Life Sci* 2007; **80**: 842-851.
19. Bredt DS, Snyder SH. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc Nat Acad Sci* 1990; **87**: 682-685.
20. Bäck M, Airila-Manson S, Jogestrand T, Söder B, Söder P. Increased leukotriene concentration in gingival crevicular fluid from subjects with periodontal disease and atherosclerosis. *Atherosclerosis* 2007; **193**: 389-394.
21. Smith MA, Braswell LD, Collins JG, Boyd DL, Jeffcoat MK, Reddy M, Li KL, Wilensky S, Vogel R, Alfano M, Offenbacher S. Changes in inflammatory mediators in experimental periodontitis in the rhesus monkey. *Infec Immun* 1993; **61**: 1453-1459.
22. Hettmannsperger U, Detmar M, Owsianowski M, Tenorio S, Kammler HJ, Orfanos CE. Cytokine-stimulated human dermal microvascular endothelial cells produce interlukin 6-inhibition by hydrocortisone, dexamethasone and calcitriol. *J Invest Dermatol* 1992;**99**: 531-536.
23. Kim SJ, Choi EY, Cho YJ, Lee JY, Choi JI, Choi IS. Surface-associated material from *Porphyromonas gingivalis* stimulates the release of nitric oxide by inducing expression of inducible nitric oxide synthase. *Microb Infect* 2006; **8**: 470-477.

24. Solito E, Parente L. Modulation of phospholipase A2 in human fibroblasts. *Br J Pharmacol* 1989; **96**: 656-660.
25. Matejka M, Partyka L, Ulm C, Solar P, Sinzinger H. Nitric oxide synthesis is increased in periodontal disease. *J Periodont Res* 1998; **33**: 517-518.
26. Lappin DF, Kjeldsen M, Sander L, Kinane DF. Inducible nitric oxide synthase expression in periodontitis. *J Periodont Res* 2000; **35**: 369-373.
27. Buckle AD, Parker SJ, Bloom SR, Edwards AV. The role of nitric oxide in the control of protein secretion in the submandibular gland of the cat. *Exp Physiol* 1995; **80**: 1019-1030.
28. Rosignoli F, Roca V, Meiss R, Pregi N, Pérez Leirós C. Inhibition of calcium-calmodulin kinase restores nitric oxide production and signaling in submandibular glands of a mouse model of salivary dysfunction. *Br J Pharmacol* 2004; **143**: 1058-1065.
29. Zhong Y, Slade GD, Beck JD, Offenbacher S. Ginvival fluid interleukin-1 beta, prostaglandin E_2 and periodontal status in a community population. *J Clin Periodonotol* 2007; **34**: 285-293.
30. Yu YH. Modulating effects of prostaglandins on parasympathetic mediated secretory activity at rat salivary gland. *Prostaglandins* 1986; **31**: 1087-1097.
31. Lathi A, Lahde M, Kankaanranta H, Moilanen E. P38 mitogen-activated protein kinase inhibitor SB203580 has a bidirectional effect on i-NOS expression and NO production. *Eur J Pharmacol* 2002; **454**: 115-123.
32. De la Cal C, Lomniczi A, Mohn CE, De Laurentiis A, Casal M, Chiarenza A, Paz D, McCann SM, Rettori V, Elverdín JC. Decrease in salivary secretion by radiation mediated by nitric oxide and prostaglandins. *Neuroimmunomodulation* 2006; **13**: 19-27.
33. Bai CH, Song SY, Kim YD. The inhibitory effect of the leukotriene receptor antagonist on leukotriene D_4-induced *MUC2/5AC* gene expression and mucin secretion in human airway epithelial cells. *Auris Nasus Larynx* 2007; **34**: 203-206.
34. Curry CC, Barbieri EJ. The modulatory effects of leukotrienes C4 and D4 on methacholine- and substance P-induced salivary secretion in rats. *Prostaglandins* 1988; **35**: 149-162.

35. Gosselin J, Borgeat P, Flamand L. Leukotriene B_4 protects latently infected mice against murine cytomegalovirus reactivation following allogeneic transplantation. *J Immunol* 2005; **174**: 1587-1593.
36. Demitsu T, Katayama H, Saito-Taki T, Yaoita H, Kakano m. Phagocytosis and bactericidal action of mouse peritoneal macrophages treated with leukotriene B_4. *Int J Immunopharmacol* 1989; 11: 801-808.
37. Leher RL, Ganz T. Defensins of vertebrate animals. *Curr Opin Immunol* 2002; **14**: 96-102.
38. Tao R, Jurevic RJ, Coulton KK, Tsutsui MT, Roberts MC, Kimball JR, Wells N, Berndt J, Dale BA. Salivary antimicrobial peptide expression and dental caries experience in children. *Antimicrob Agents Chemother* 2005; **9**: 3883-3888.
39. Tabak LA. Structure and function of human salivary mucins. *Oral Biol Med* 1990; **1**: 229-234.
40. Snyder DW, Fleisch JH. Leukotriene receptor antagonists as potential therapeutic agents. *Annu Rev Pharmacol Toxicol* 1989; **29**: 123-143.
41. Dahlén SE. Pharmacological characterization of leukotriene receptors. *Am J Crit Care Med* 2000; **161**: 541-545.
42. Boehm S, Kubista H. Fine tuning of sympathetic transmitter release via ionotropic and metabotropic presynaptic receptors. *Pharmacol Rev* 2002; **54**: 43-99.
43. Stanke-Labesque F, Hardy G, Caron F, Cracowski JL, Bessard G. Inhibition of leukotriene synthesis with MK-886 prevents a rise in blood pressure and reduces noradrenaline-evoked contraction in L_NAME-treated rats. *J Pharmacol* 2003; **140**: 186-194.

Table 1: Basal mucin release in the absence and presence of the cysteinyl leukotriene receptor antagonist, FPL 55712, in submandibular gland from control and with experimental periodontitis rats.

Group	Basal mucin release (% of total)

Control	5.24 ± 0.74
Periodontitis	8.28 ± 0.27[a]
Control + FPL	4.52 ± 0.71
Periodontitis + FPL	4.90 ± 0.61

Mucin secretion was measured in the absence and the presence of FPL 55712 10^{-8} M in submandibular gland from rats with ligature and control. a: significantly different from all other groups. $P < 0.001$.

Table 2: Body weights, submandibular glands weights and mucin concentration from rats with experimental periodontitis and controls.

Group	Rat body weight g	Gland weight (mg)	Mucin (μg/ mg) #

Control	300 ± 12	200 ± 3.8 (n: 30)	33.0 ± 2.6 (n: 10)
Periodontitis	310 ± 15	230 ± 3.6[a](n: 30)	24.7 ± 1.4[c] (n: 10)
Control + NDGA	305 ± 10	209 ± 4.0 (n: 8)	32.4 ± 3.0 (n: 4)
Periodontitis + NDGA	300 ± 16	228 ± 4.8 [b] (n: 8)	31.1 ± 2.8[d] (n: 4)

Rat body and submandibular gland weights were checked before the experiment. #Submandibular glands from rats with ligature and control were incubated in the absence and the presence of NDGA 5 x 10^{-6} M and mucin content in the gland after the incubation period (residual mucin) was determined as stated in Methods.

a and b: significantly different from control group. P< 0.001 and P< 0.01 respectively. c: significantly different from control group. P< 0.05. d: significantly different from periodontitis group without NDGA. P< 0.05.

Legends of Figures

Figure 1: Panel A: Effect of hydrocortisone, given sc 1 mg/ k during the last 3 days before experiments, on basal mucin secretion by submandibular gland from control and with experimental periodontitis rats. Bars represent the mean ± SEM of 6 experiments. ** Significantly different from basal with periodontitis. P< 0.01. Panel B: Effect of the β-adrenoceptors antagonists, propranolol and atenolol, both at 5 x 10^{-7} M, on non-

stimulated mucin release by submandibular glands from control and with experimental periodontitis rats. Bars represent the mean ± SEM of 4 experiments. * Significantly different from basal with periodontitis. $P< 0.05$.

Figure 2: Panel A: Nitric oxide synthase activity in submandibular gland from control and with experimental periodontitis rats. Submandibular gland from control and with experimental periodontitis rats were incubated with L (U-^{14}C)-arginine and the activity of NOS was determined as described in Methods. Assays were carried out in the absence (basal) and the presence of 5 x 10^{-6} M L-NMMA as indicated. Bars represent the mean ± SEM of 4 experiments. * Significantly different from basal, $P< 0.05$. Panel B: Effect of L-NMMA on isoproterenol-induced mucin secretion in submandibular gland from control and with experimental periodontitis rats. Submandibular gland from control and with experimental periodontitis rats were incubated with increasing concentrations of isoproterenol in the absence and the presence of 5 x 10^{-6} M L-NMMA and mucin secretion was determined as stated in Methods. Each point represents the mean ± SEM of 4 experiments. ● Control group; ○ Periodontitis group; ▼ Control group with L-NMMA; ∇ Periodontitis group with L-NMMA.

Figure 3: Panel A: PGE_2 production by submandibular gland from control and with experimental periodontitis rats. Submandibular gland from control and with experimental periodontitis rats were incubated in the absence (basal) and the presence of 5 x 10^{-6} M indomethacin or 10^{-8} M DuP 697 as indicated, and PGE_2 was determined as stated in Methods. Bars represent the mean ± SEM of 4 experiments. *** Significantly different from basal, $P< 0.001$. Panel B: Effect of indomethacin on isoproterenol-induced mucin secretion in submandibular glands from control and with experimental periodontitis rats. Submandibular gland from control and with experimental periodontitis rats were incubated with increasing concentrations of isoproterenol in the absence and the presence of 5 x 10^{-6} M indomethacin and mucin secretion was determined as stated in Methods. Each point represents the mean ± SEM of 4 experiments. ● Control group; ○ Periodontitis group; ▲ Control group with indomethacin; △ Periodontitis group with indomethacin.

Figure 4: Panel A: Concentration of cysteynil leukotrienes in submandibular gland from control and with experimental periodontitis rats. Submandibular glands from control and with experimental periodontitis rats were incubated in the absence (basal) and the presence of 5 x 10^{-6} M NDGA and cysteynil leukotrienes were determined as stated in

Methods. Bars represent the mean ± SEM of 4 experiments. *** Significantly different from all other groups. P< 0.001. Panel B: Effect of NDGA on isoproterenol-induced mucin secretion in submandibular gland from control and with experimental periodontitis rats. Submandibular glands from control and with experimental periodontitis rats were incubated with increasing concentrations of isoproterenol in the absence and the presence of 5 x 10^{-6} M NDGA and mucin secretion was determined as stated in Methods. Each point represents the mean ± SEM of 4 experiments. ● Control group; ○ Periodontitis group; ◆ Control group with NDGA; ◇ Periodontitis group with NDGA

Figure 5: Hypothetic events that provoke an increase in mucin release in submandibular gland from rats with experimental periodontitis.