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β-Adrenoceptor alterations coupled with secretory response and experimental periodontitis in rat submandibular glands

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ABSTRACT

In this paper we have studied the influence of a well-established rat model of periodontitis on resting and adrenergic-stimulated mucin secretion from rat submandibular glands. The selective β_1-receptor subtype agonist, dobutamine, induced mucin secretion while the selective β_2-, α_1- and α_2-agonists, soterenol, phenylephrine and clonidine, respectively, did not. In rats subjected to ligature-induced periodontitis mucin release, under unstimulated conditions (basal values), was significantly increased. This increment was abolished in the presence of propranolol and atenolol. Isoproterenol, concentration-dependent, increased mucin release in control and in ligature-induced periodontitis rats. Maximal effect of isoproterenol was decreased in rats with ligature while EC_{50} was increased. Neither, the inhibition of NOS by L-NMMA nor the inhibition of COX by indomethacin could revert the effect of ligature on mucin release under unstimulated and isoproterenol-stimulated conditions. The inhibition of adenylyl cyclase by SQ 22536 resulted in a right shift of isoproterenol concentration–response curves in both groups, control and with ligature and returned basal values of rats with ligature to control ones. β-Receptor population was decreased in submandibular gland membranes from rats with ligature without changes in affinity. Potencies of the β-receptor antagonists in the competition studies were similar in both groups under study, control and with ligature. We conclude that in rats subjected to ligature-induced periodontitis unstimulated mucin secretion is increased. The increment seems to be due to an activation of the sympathetic system since it is inhibited by the β-adrenoceptors antagonists and by the inhibition of the adenylyl cyclase. We can speculate that inflammatory mediators from the experimental periodontitis could be involved in the mechanism underlying the activation of the sympathetic system.

1. Introduction

Human periodontal disease is an inflammatory disorder that gives rise to tissue damage and loss as a result of complex interaction between pathogenic bacteria and the host's immune response. This disease is primarily related to chronic plaque accumulation. Putative periodontopathic bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia* or *Actinobacillus actinomycetemcomitans* are suspected to play a role in the periodontal disease process.[1] Periodontitis, affecting 7–15% of the adult population, is a multi-factorial disease in which the existence of pathogenic bacteria is necessary but not sufficient. Under the influence of several behavioural, environmental and genetic factors, the host immunologic and

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inflammatory response is the critical determinant of susceptibility to the disease.[2]

Salivary flow and proteins, including immunoglobulins and mucins, are believed to support clearance of micro-organisms from the oral cavity. Oral disease, including periodontal infections, can occur when such defence mechanisms are impaired. Saliva has been demonstrated to inhibit the adhesion of *A. actinomycetemcomitans* to oral epithelial cells.[3] Salivary components that interact with *A. actinomycetemcomitans* include fractions of S-IgA, lactoferrin and the low M_r mucin species MG2.[4,5] Salivary mucins are heavily glycosylated high molecular weight glycoproteins produced by various (sero) mucous salivary glands, i.e. submandibular, sublingual and palatal glands and the minor salivary glands in the lip, cheek and tongue. Mucins play a major role in the maintenance of viscoelastic properties of saliva, participate in the formation of protective oral mucosal mucus coat and tooth enamel pellicle,[6] promote bacterial aggregation and clearance from the oral cavity[7] and as shown recently, are capable of modulating the process occurring within the epithelial perimeter of oral mucosal defence, such as receptor-gated ion channel activity.[8]

The salivary glands are innervated by sympathetic and parasympathetic nerves, which regulate secretion of water, electrolytes and proteins. Parasympathetic stimulation leading to muscarinic cholinergic receptor activation is linked to formation of inositol triphosphate and diacylglycerol and subsequent rise in intracellular calcium, which open membrane ion channels, most notably apical chloride channels, leading to fluid secretion.[9] However, cholinergic stimuli can give rise to the release of protein in part by the release of the neuropeptide co-transmitter vasointestinal polypeptide (VIP) but also, by a coupling mechanism involving elevated intracellular calcium and activation of protein kinase C.[9] Noradrenaline released from sympathetic nerves stimulates salivary secretion through α_1- and β_1-adrenoceptors. Alpha$_1$-adrenoceptors-mediated signals follow a similar pathway as described for parasympathetic stimulation leading to fluid secretion, while β_1-adrenoceptors signalling mainly occurs through G-protein/adenylate cyclase generation of intracellular cAMP followed by activation of protein kinase A and phosphorylation of endogenous protein leading to exocytosis of protein storage granules and salivary protein secretion.[9] In the rat submandibular gland, stimulation of the β-adrenergic receptors results in extensive mucus secretion.[10] Taken together, the importance of mucin to the protection of oral mucosal and soft gingival tissue from mechanical, chemical and microbial insults and the regulatory action of β-adrenergic receptor on mucin release, the aim of the present study was to evaluate whether the pattern of mucin release under resting and β-stimulated conditions was influenced by the periodontal disease induced experimentally in the rat.

2. Methods

2.1. Surgical procedure

Male Wistar rats weighing 250–300 g were lightly anaesthetised with a mixture of Ketamine and Xilazine (50 and 5 mg/kg, respectively). A black thread was placed around the cervix of both, left and right, lowers first molars and knotted mesially.[11] Experiments were carried out 22 days after the rats were subjected to ligature-induced periodontitis. For histopathological examination, biopsies of gingivomucosal tissue were taken 22 days after induction of ligature-induced periodontitis. The tissues slices were fixed in 10% neutral-buffered formaldehyde for 5 days embedded in paraffin and sectioned. The sections were stained with haematoxylin and eosin. The total number of infiltrating leucocytes (e.g., neutrophils and mononuclear cells) in cortical interstitial spaces was assessed quantitatively by counting the number of polymorphonuclear cells in 20 high-power fields. In rats subjected to ligature-induced periodontitis the presence of oedema, tissue injury and a large number of infiltrating polymorphonuclear cells in the gingivomucosal tissue, confirmed the presence of an inflammatory process. 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).

2.2. 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 were included from the beginning of the incubation and stimuli were added in the last 15 min of the incubation time. Placing the tubes on ice then stopped the reaction. Glands were homogenised in 50 mM sodium acetate buffer, 25 mM Cl_2Mg, pH 5.8, supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium ethylenediaminetetra-acetate and 1 mM iodoacetamide) at 4 °C and centrifuged at 900 × *g* for 15 min. Mucin was determined in the supernatants (total mucin content in the gland) and in the incubation medium (mucin released) using the Alcian Blue method described by Hall et al.[12] and modified by Sarosiek et al.[13] 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 (Docusate sodium salt, SigmaUltra, minimum 99%, 10% solution, Sigma Chemical Company, 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 mucin content in the gland (% of total).[14]

2.3. *In vivo experiments*

Rats received vehicle or indomethacin 1 mg/kg, intraperitoneally (daily treatment for 3 days) and the measurement of mucin release was performed as stated above.

2.4. *Radioligand binding assays*

Submandibular glands were freed of connective tissue, fat and lymph nodes and then homogenised in Ultraturrax in 10 mM Tris buffer (pH 7.4) with 10 mM Cl_2Mg and 25 mM sacarose, supplemented with the following protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride, 2 $\mu g\,ml^{-1}$ leupeptin, 1 $\mu g\,ml^{-1}$ aprotinin, 1 mM sodium ethylenediaminetetra-acetate (EDTA), 2 μM pepstatin A and 1 mM iodoacetamide. The slurry was then centrifuged twice for 15 min at 1000 × *g*, once for 30 min at 12,000 × *g* and then for 90 min at 40,000 × *g*, at 4 °C to recover a plasma membrane-enriched fraction. For binding experiments, the plasma membrane-enriched fraction was resuspended in 50 mM Tris buffer (pH 7.4), with the same protease inhibitors at a concentration of approximately 500 $\mu g\,ml^{-1}$. Saturation assay was determined by incubation of 100 μg of protein with 0.25–32 nM of the β_1-adrenergic receptor antagonist [^{3}H]-CGP in a total volume of 150 μl for 30 min at 37 °C with continuous shaking. Binding was stopped by adding 2 ml of ice-cold buffer followed by rapid filtration (Whatman GF/c). Filters were rinsed with 4 ml of ice-cold buffer, transferred into vials containing 1 ml of Scintillation cocktail (Optiphase ''Hisafe'' 3) and counted in a liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 10 μM propranolol. For competition binding assay, membranes (100 μg protein) were incubated with 4 nM [^{3}H]-CGP and increasing concentration of antagonists and the experiments were carried out as described above.

2.5. *Drugs*

Isoproterenol, propranolol, dobutamine, atenolol, soterenol, phenylephrine, clonidine, atenolol, butoxamine, L-NMMA and indomethacin are from Sigma Chemical Company (St. Louis, MO, USA). SQ 22536 was purchased from Research Biochemicals International (Natick, MA, USA).

2.6. *Statistical analysis*

Statistical significance of differences was determined by analysis of variance (ANOVA) followed by Tukey's test. Unpaired *t*-test was used for comparing two groups. Differences between means were considered significant at $P < 0.05$. Fitting dose–response curves, saturation curves and Scatchard plot were done using GraphPad Prism Version 4.00 for Windows (GraphPad Software, San Diego CA, USA).

3. Results

We first characterised the adrenergic receptor involved in mucin secretion in submandibular gland. The selective β_1-receptor agonist, dobutamine increased mucin secretion while the selective β_2, α_1 and α_2 receptor agonists, soterenol, phenylephrine and clonidine, respectively, failed in increasing mucin secretion from the submandibular gland (Table 1). Concentration–response curve to isoproterenol on mucin secretion was right shifted in the presence of the selective β_1-receptor subtype antagonist, atenolol, resulting in an increase of EC_{50} values from 8.3×10^{-9} to 1.4×10^{-7} M ($p < 0.01$) confirming the participation of the β_1-receptor subtype in mucin secretion. The selective β_2-receptor subtype antagonist, butoxamine, lacked of inhibitory effect (EC_{50} in its presence: 6.2×10^{-9} M).

No sign of inflammation was observed in submandibular glands from rats with ligature but gland weights were increased in about 10% (control: 204.8 ± 3.8; ligature: 224.8 ± 3.6, *n*: 30, $p < 0.001$) while the absolute levels of mucin were decreased in 18% (control: 38.7 ± 1.4; ligature 31.7 ± 1.4, *n*: 30, $p < 0.001$).

Fig. 1 shows that mucin release in response to isoproterenol was influenced by the ligature-induced periodontitis. The maximal effect was decreased (14.4 ± 0.47 and 11.82 ± 0.40 for control and ligature, respectively, $p < 0.01$) and the EC_{50} value was increased (1.02×10^{-8} and 1.11×10^{-6} M for control and ligature, respectively, $p < 0.001$). Conversely, in resting conditions (basal values) mucin release was significantly higher than in control animals (5.8 ± 0.3 and 8.0 ± 0.6 for control and ligature, respectively, $p < 0.05$).

The non-selective β-receptor antagonist, propranolol, as well as the β_1-selective receptor antagonist, atenolol, reduced

Table 1 – Submandibular gland secretory response to increasing concentration of the selective adrenoceptor agonists dobutamine, soterenol, clonidine and phenylephrine

Agonist	Concentration of the agonist			
	None (M)	10^{-8} M	10^{-7} M	10^{-6} M
Dobutamine	5.6 ± 0.5	7.4 ± 0.6	9.7 ± 1.0**	11.2 ± 1.1**
Soterenol	6.0 ± 0.6	6.8 ± 0.5	6.7 ± 0.7	5.1 ± 0.6
Clonidine	5.6 ± 0.6	6.6 ± 0.6	5.3 ± 0.5	5.5 ± 0.6
Phenylephrine	5.2 ± 0.7	6.4 ± 0.5	6.0 ± 0.5	5.3 ± 0.6

Submandibular slices were incubated in the absence or the presence of the indicated concentrations of the β_1-selective agonist, dobutamine, the β_2-selective agonist, soterenol, the α_2-selective agonist, clonidine, and the α_1-selective agonist, phenylephrine and mucin release was measured as described in Section 2. Data are expressed as percentage release from total mucin content in the gland. Values are the mean ± S.E.M. of four independent experiments. **$p < 0.01$ vs. basal values.

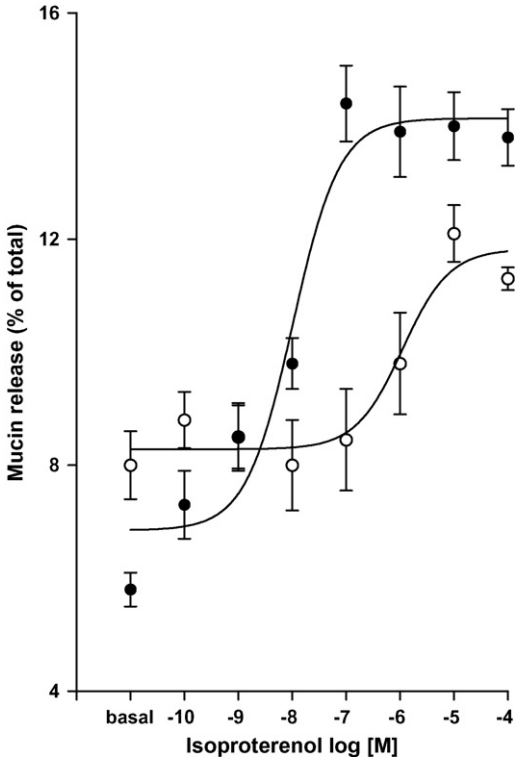

Fig. 1 – Effect of isoproterenol on mucin release in submandibular glands from control (●) and from rats subjected to ligature-induced periodontitis (○). Results are expressed as percentage released from total mucin content in the gland. Each point represents the mean ± S.E.M. of four independent experiments.

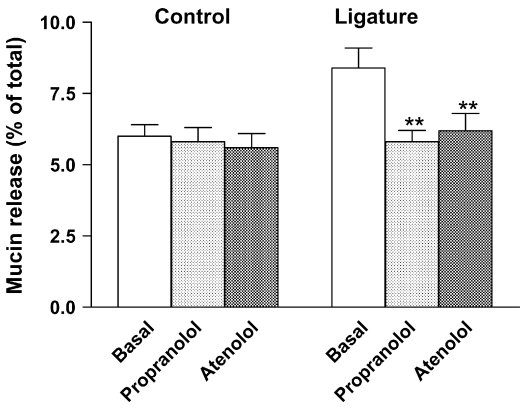

Fig. 2 – Effect of propranolol and atenolol on mucin secretion in resting conditions in submandibular glands from control and from rats subjected to ligature-induced periodontitis. Submandibular slices were incubated with vehicle or in the presence of 5×10^{-7} M propranolol or 5×10^{-7} M atenolol. Results are expressed as percentage released from total mucin content in the gland. Each bar represents the mean ± S.E.M. of four independent experiments.

mucin release under resting conditions from rats with ligature-induced periodontitis reaching values similar to that of controls (Fig. 2). The antagonist drugs did not change basal values from control rats (Fig. 2).

The adenylyl cyclase (AC) inhibitor, SQ 22536 (5×10^{-6} M), abolished the effect of ligature-induced periodontitis on basal values and inhibited isoproterenol-induced mucin release in both groups control and with ligature (Fig. 3A and B).

The evaluation of β-adrenergic receptors revealed that in gland membranes from rats subjected to ligature-induced periodontitis the receptor population decreased without change the affinity (Fig. 4, upper panel). The data obtained from binding studies using the β_1-receptor antagonist [^{3}H]-CGP showed that the maximal binding sites decreased from 198.9 ± 12 to 112.8 ± 4 fmol/mg protein ($p < 0.001$) with no change in the K_d values, 1.23 ± 0.16 nM for gland membranes from rats with ligature and 1.02 ± 0.23 for controls (Fig. 4, upper panel, A and B).

The lower panel of Fig. 4 shows the competition binding assays of β-adrenergic receptor antagonists with [^{3}H]-CGP in submandibular membranes from rats with ligature and controls. As can be seen, the non-selective β-receptor antagonist, propranolol, and the selective β_1-receptor antagonist, atenolol, shifted the ligand in both groups, control and with ligature, with K_i values about 10^{-7} M (Fig. 4, lower panel, A and B). The selective β_2-receptor antagonist, butoxamine, failed in the competition assay (Fig. 4, lower panel C).

Fig. 5A shows that the presence of the nitric oxide synthase (NOS) inhibitor, L-NMMA 10^{-5} M, did not revert the effect of the ligature-induced periodontitis on mucin release under unstimulated and isoproterenol-stimulated conditions.

The in vivo effect of indomethacin, which was given intraperitoneally 1 mg/kg during 3 days, is shown in Fig. 5B. As can be seen the inhibition of the cyclooxygenase failed to modify the mucin secretory pattern in submandibular glands from rats subjected to ligature-induced periodontitis. In addition, basal values were not modified by indomethacin, 5×10^{-6} M, when was included at the beginning of the incubation time (data not shown).

4. Discussion

In this paper we have studied the influence of a well-established rat model of periodontitis on the resting and adrenergic-stimulated mucin secretion from submandibular glands.

Our results show that the β_1-adrenergic receptor subtype is the responsible for mucin secretion in the rat submandibular gland. Our selection of the non-selective β-receptor agonist, isoproterenol, was based on the fact that it is the drug of choice for experimental studies.

Resting and stimulated mucin secretion showed changes in rats subjected to ligature-induced periodontitis. Mucin release during resting conditions was increased while isoproterenol-stimulatory action was decreased. Since resting conditions prevail in the oral cavity for most of a 24-h period, changes in mucin basal values are of most interest.

In rats subjected to ligature-induced periodontitis, the presence of the β-adrenergic receptor antagonists, proprano-

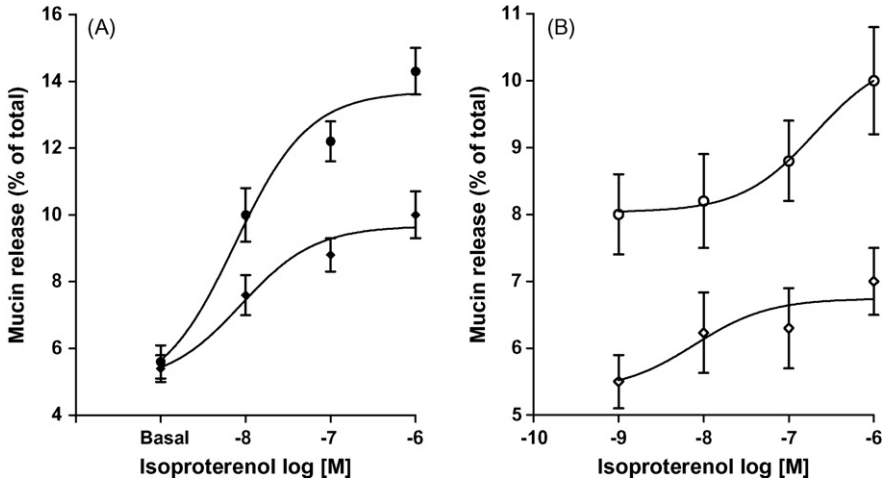

Fig. 3 – Effect of SQ 22536 on mucin release under unstimulated and isoproterenol-stimulated conditions in submandibular glands from control (A) and from rats subjected to ligature-induced periodontitis (B). Submandibular slices were incubated with vehicle or the indicated concentration of isoproterenol alone (●, A) and (○, B) and in the presence of 5×10^{-6} M SQ 22536 (◆, A) and (◇, B). Results are expressed as percentage released from total mucin content in the gland. Each point represents the mean ± S.E.M. of four independent experiments.

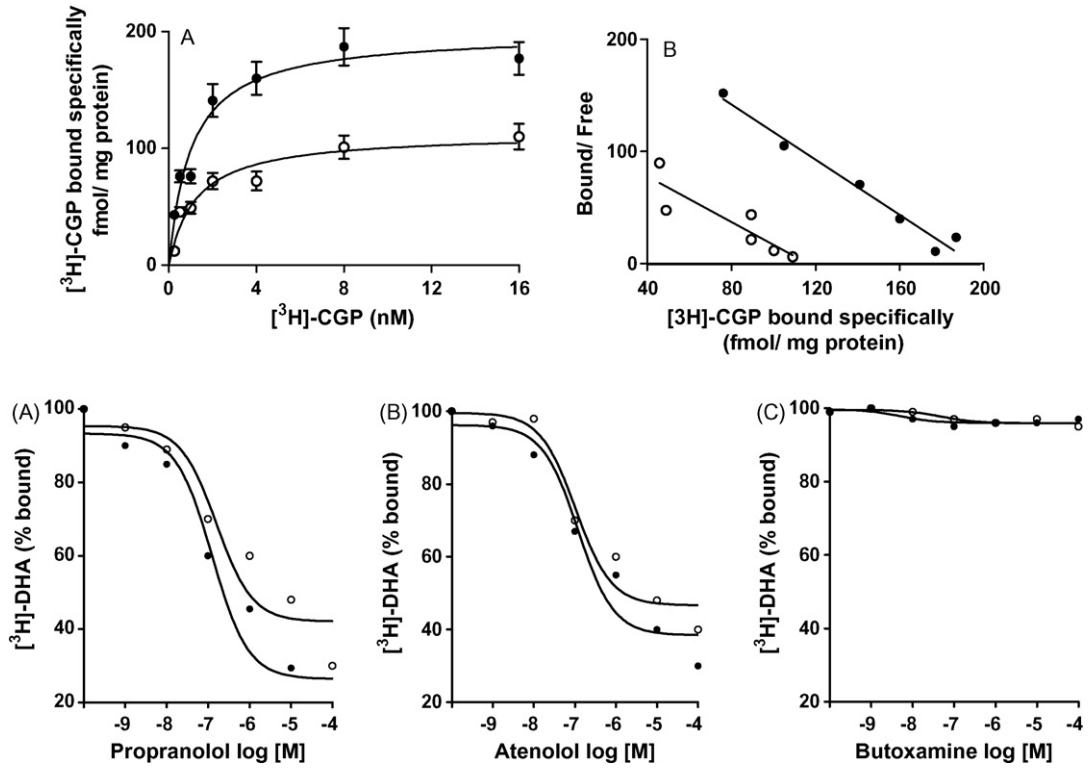

Fig. 4 – Upper panel: saturation curves (A) and Scatchard plots (B) on submandibular gland membranes from control (●) and from rats subjected to ligature-induced periodontitis (○). Submandibular gland membranes were incubated with different concentration of [^{3}H]-CGP and binding assay was performed as stated in Section 2. Results are the mean ± S.E.M. of four independent experiments performed in duplicate in each group. Lower panel: competition binding assays of the β-adrenergic antagonists with [^{3}H]-CGP in submandibular gland membranes from control (●) and from rats subjected to ligature-induced periodontitis (○). Submandibular gland membranes were obtained and competition binding assays performed as indicated in Section 2 with different concentrations of propranolol (A), atenolol (B) and butoxamine (C). Results shown are representative of three independent experiments performed in duplicate with similar results.

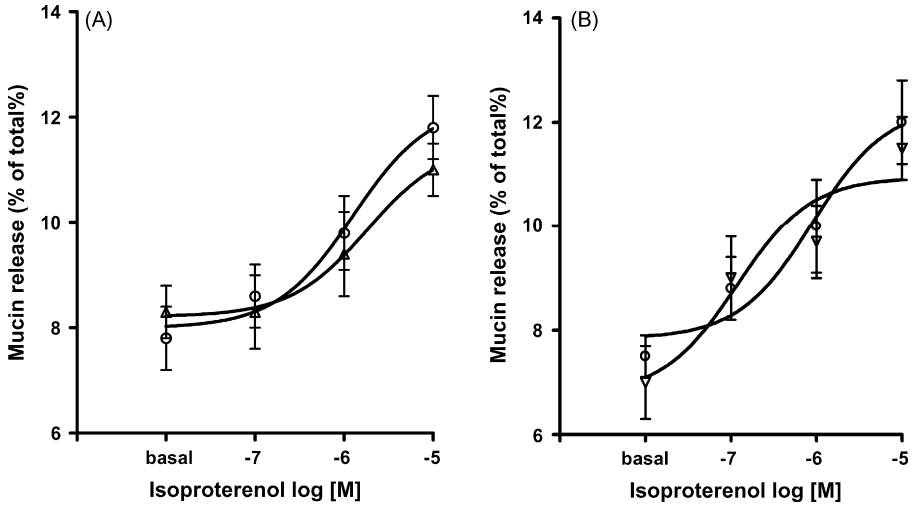

Fig. 5 – Panel A: effect of L-NMMA on mucin release under unstimulated and isoproterenol-stimulated conditions in submandibular glands from rats subjected to ligature-induced periodontitis. Submandibular slices were incubated with vehicle or the indicated concentrations of isoproterenol alone (○) and in the presence of 10^{-5} M L-NMMA (△). Results are expressed as percentage released from total mucin content in the gland. Each point represents the mean ± S.E.M. of four independent experiments. Panel B: *ex vivo* effect of indomethacin (1 mg/kg, i.p. 3 days) on mucin release under unstimulated and isoproterenol-stimulated conditions, in submandibular glands from rats subjected to ligature-induced periodontitis. Submandibular slices from rats pre-treated with vehicle (○) or indomethacin (▽) were incubated with vehicle or the indicated concentrations of isoproterenol. Results are expressed as percentage released from total mucin content in the gland. Each point represents the mean ± S.E.M. of four independent experiments.

lol and atenolol, resulted in an inhibition of the increase mucin secretion under resting conditions, leaving the values to control ones. This result indicates that the sympathetic system is involved in the increased mucin secretion observed in rats with ligature-induced periodontitis. In control animals the presence of β-antagonists did not modify basal mucin secretion, because in resting conditions there is a constitutive secretion of protein storage in granules.[15] As the salivary secretion is a reflex response controlled by both parasympathetic and sympathetic secretomotor nerves it can be influenced by several stimuli. Among the stimuli involved in salivary reflex are grooming, eating, heat,[16] gustatory and olfactory impulses[17] and, as was demonstrated in human studies, activation of periodontal mechanoreceptors.[18] Studies of reflex secretion from submandibular glands in conscious rats have shown that outputs of saliva differ with different afferent stimulations.[19] In addition, the contribution of sympathetic and parasympathetic nerves during such reflex activities is different, depending on the different stimuli-induced secretion.[16] In experimental gingivitis, the salivary gland output increases and this finding was attributed to the accumulation of plaque-derived substances or inflammatory products that triggers the salivary secretion via neural pathways.[20] It was described that, prior exposure of submandibular acini to isoproterenol for 45 min, followed by washout, resulted in persistent increase in basal secretion, which was abolished by propranolol.[21] In line with this, and considering that in slide preparations nerves are still attached to cells, we could detect an increase of sympathetic activity though our experimental model comprised isolated submandibular glands. As a matter of fact submandibular gland weights from rats with ligature were significantly increased as happen when salivary glands are continuously stimulated and in concordance with this, absolute levels of mucin were decreased. Changes in the glandular output of proteins during periodontal inflammation have been reported in patients with juvenile periodontitis, who displayed increased rates of salivary epidermal growth factor (EGF) secretion.[22] Cystatins and IgA, both acting as important first line of defence mechanism in the oral cavity, are increased in inflammatory periodontal disease.[23,20] It is important to note that EGF, cystatins and IgA secretion is under sympathetic regulation.[24–26] This fact strongly supports the view that sympathetic activity is increased during periodontal disease, although proteins can be also secreted by parasympathetic nerves.[9]

In rats subjected to ligature-induced periodontitis low concentrations of isoproterenol failed in increasing mucin release over basal values. Exposure of rat submandibular acini to isoproterenol desensitises the cells to subsequent stimulations.[21] Thus, submandibular glands of rats with ligature that are exposed continuously to sympathetic stimulation needed higher concentrations of isoproterenol for increasing mucin release from basal values.

The inhibition of adenylyl cyclase by SQ 22536 impaired isoproterenol-induced mucin secretion in control and subjected to ligature-induced periodontitis rats, indicating that ligature did not modify the cAMP-mediating mechanism of action of isoproterenol. In addition, SQ 22536 abolished the increment of basal values induced by ligature, result that is in line with the participation of β-adrenergic activation in this process.

Binding studies showed that β_1-adrenergic receptor expression was diminished in sites in submandibular glands from rats subjected to ligature-induced periodontitis, without

any alteration in the equilibrium dissociation constant. Decrease number of β-adrenoceptors in parotid gland was observed after long periods of incubation with isoproterenol.[27] Thus, the continuous presence of the sympathetic agonist in submandibular glands from rats with ligature induced a down-regulation of β-adrenoceptors.

Potencies of the antagonists in displacing [^{3}H]-CGP from submandibular membranes were similar in both groups under study, controls and with ligature. This result supports the view that ligature-induced periodontitis only affects β-adrenoceptors sites without changing the affinity.

Nitric oxide (NO) in periodontal tissue and saliva may be part of the nonspecific natural defence mechanism of the oral cavity against pathogenic bacteria or alternatively, excessive amounts of NO may contribute to tissue destruction in periodontitis.[28] This intracellular and diffusible messenger is an inflammatory mediator in salivary glands diseases[29] and it was found that NO triggers the activation of cyclooxygenase-2 (COX-2).[30] Enhanced production of NO has been demonstrated in periodontal diseases.[31] In submandibular glands it has been suggested that NO is involved in blood flow and protein secretion as inhibitors of NO synthesis abolished nerve-evoked protein output.[32] To investigate the possible involvement of NO in the changes observed in mucin secretion under resting and stimulated conditions, we studied the effect of the NOS inhibitor L-NMMA in submandibular glands from rats subjected to ligature-induced periodontitis. Our results suggest that, though NO production could be increased in rats subjected to ligature-induced periodontitis, this messenger is not responsible for the alteration pattern of mucin secretion.

The participation of prostaglandins (PGs) during gingivitis and periodontal disease has been well documented. Patients with chronic periodontitis show increased PGE_2 levels[33,34] and endotoxin-induced periodontitis in rat's results in significant elevations of PGE_2 in the gingival tissue.[35] On the other hand, PGs are related with mucin synthesis and release in the guinea pig antral mucous cells[36] and in rat submandibular gland.[14] Taken together, we investigate the effect of the inhibition of cyclooxygenase, by intraperitoneal administration of indomethacin, on unstimulated and stimulated mucin secretion in submandibular glands from rats subjected to ligature-induced periodontitis. Our results demonstrate that the cyclooxygenase products are not involved in the signalling pathway that increase mucin release during resting conditions or in the altered pattern of isoproterenol-induced mucin secretion.

In conclusion we demonstrate here that ligature-induced periodontitis induces an increase of unstimulated mucin secretion from submandibular glands. In control rats unstimulated mucin secretion is constitutive, but in periodontitis the increase of mucin secretion seems to be the result of an activation of the sympathetic system. This hypothesis is supported by the following observations: first, the presence of the β-adrenoceptors antagonists, propranolol and atenolol inhibited the increment of mucin basal values in rats with ligature. Second, the increase of secretion is achieved through the activation of the adenyl cyclase, the same mechanism underlying β-adrenoceptors activation. Third, in rats with ligature it was observed a desensitation to isoproterenol stimulation and a decrease in β-adrenoceptors expression, as happen when a receptor is continuously stimulated. Neither nitric oxide synthase nor cyclooxygenase are involved in the mechanism underlying mucin secretion in rats with ligature. We can speculate that inflammatory mediators different from nitric oxide and prostaglandins could be involved in the mechanism underlying the activation of the sympathetic system in rats with ligature.

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REFERENCES

1. Henskens YMC, Van den Keijbus PAM, Veerman ECI, Vand der Weijden GA, Timmerman MF, Snoek CM, et al. Protein composition of whole and parotid saliva in healthy and periodontitis subjects. *J Periodontal Res* 1996;**31**:57–65.
2. 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.
3. Mintz KP, Fives-Taylor PM. Adhesion of *Actinobacillus actinomycetemcomitans* to a human oral cell line. *Infect Immun* 1994;**62**:3672–8.
4. Alugupalli KR, Kalfas S, Eswardsson S, Naidu AS. Lactoferrin interaction with *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol* 1995;**10**:35–41.
5. Groenink J, Ligtenberg AJM, Veerman ECI, Bolsher JGM, Nieuw Amerongen AV. Interaction of the salivary low-molecular-weight mucin (MG2) with *Actinobacillus actinomycetemcomitans*. *Antonie van Leeuwenhoek* 1996;**70**: 79–87.
6. Slomiany A, Okazaki K, Tamura S, Slomiany BL. *In vitro* sulfation of sublingual salivary gland mucin: structures of ^{15}S-labeled oligosaccharides. *Biochem Int* 1989;**18**:589–97.
7. Tabak LA. Structure and function of human salivary mucins. *Oral Biol Med* 1990;**1**:229–34.
8. Slomiany BL, Fekete Z, Murty VL, Grabska M, Piotrowski J, Yotsumoto F, et al. Regulation of buccal mucosal calcium channel activity by salivary mucins. *Int J Biochem* 1993;**25**:1281–9.
9. Proctor GB, Carpenter GH. Regulation of salivary gland function by autonomic nerves. *Auton Neurosci* 2007;**133**:3–18.
10. Martinez JR, Quissell DO, Wood DL, Giles M. Abnormal secretory response to parasympathomimetic and sympathomimetic stimulation from the submaxillary gland of rats treated with reserpine. *J Pharmacol Exp Ther* 1975;**194**:384–95.
11. Di Paola R, Mazzon E, Rotondo F, Dattola F, Britti D, De Majo M, et al. Reduced development of experimental periodontitis by treatment with M40403, a superoxide dismutase mimetic. *Eur J Pharmacol* 2005;**516**: 151–7.
12. Hall RL, Miller AC, Peatfield PS, Richardson I, Willliams I, Lampert I. A colorimetric assay for mucus glycoprotein using Alcian blue. *Biochem Soc Trans* 1980;**8**:72–3.
13. 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. *Am J Med Sci* 1994;**308**:23–31.
14. Busch L, Borda E. Signaling pathways involved in pilocarpine-induced mucin secretion in rat submandibular glands. *Life Sci* 2007;**80**:842–51.
15. Proctor GB, Carpenter GH, Segawa A, Garrett JR, Ebersole L. Constitutive secretion of immunoglobulin A and other proteins into lumina of unstimulated submandibular glands in anaesthetized rats. *Exp Physiol* 2003;**88**:7–12.
16. Matsuo R, Garrett JR, Proctor GB, Carpenter GH. Reflex secretion of proteins into submandibular saliva in conscious rats, before and after preganglionic sympathectomy. *J Physiol* 2000;**527**:175–84.
17. Hodson NA, Linden RWA. Is there a parotid-salivary reflex response to fat stimulation in humans? *Physiol Behav* 2004;**82**:805–13.
18. Hector MP, Linden RW. The possible role of periodontal mechanoreceptors in the control of parotid secretion in man. *Q J Exp Physiol* 1987;**72**:285–301.
19. Matsuo R, Yamamoto T, Ikehara A, Nakamura O. Effects of salivation on neural taste responses in freely moving rats: analyses of salivary secretion and taste responses of the chorda tympani nerve.. *Brain Res* 1994;**649**:136–46.
20. 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**:233–7.
21. Bradbury NA, Mc Pherson MA. Isoproterenol-induced desensitization of mucin release in isolated rat submandibular acini. *Biochem Biophys Acta* 1988;**970**:363–70.
22. Hormia M, Thesleff I, 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**:138–44.
23. Henskens YMC, Veerman ECI, Mantel MS, van der Velden U, Nieuw Amerongen AV. Cystatins S and C in human whole saliva and in glandular salivas in periodontal health and disease. *J Dent Res* 1994;**73**:1606–14.
24. Lamey PJ, Savage AP, Fisher BM, Bloom SR, Frier BM. Secretion of epidermal growth factor in parotid saliva in diabetic patients: role of autonomic innervation. *J Oral Pathol Med* 1990;**19**:351–4.
25. Bedi GS. The effect of adrenergic agonists and antagonists on cysteine-proteinase inhibitor (cystatin) in rat saliva. *Arch Oral Biol* 1991;**36**:611–8.
26. Carpenter GH, Proctor GB, Ebersole LE, Garrett JR. Secretion of IgA by rat parotid and submandibular cells in response to autonomimetic stimulation in vitro. *Int Immunopharmacol* 2004;**4**:1005–14.
27. Hata F, Ishida H, Kagawa K, Kondo E, Kondo S, Noguchi Y. β-Adrenoceptor alterations coupled with secretory response in rat parotid tissue. *J Physiol* 1983;**341**:185–96.
28. Ugar-Çankal D, Ozmeric N. A multifaceted molecule, nitric oxide in oral and periodontal diseases. *Clin Chim Acta* 2006;**366**:90–100.
29. Rosignoli F, Roca V, Meiss R, Pregi N, Leiros CP. 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**:1058–65.
30. Rettori V, Lomniczi A, Elverdin JC, Suburo A, Faletti A, Franchi A, et al. Control of salivary secretion by nitric oxide and its role in neuroimmunomodulation. *Ann NY Acad Sci* 2000;**917**:258–67.
31. Matejka M, Partyka L, Ulm C, Solar P, Sinzinger H. Nitric oxide synthesis is increased in periodontal disease. *J Periodontal Res* 1998;**33**:517–8.
32. 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**: 1019–30.
33. Mizrak T, Guncu GN, Caglayan F, Balci TA, Aktar GS, Ipek F. Effect of a controlled-release chlorhexidine chip on clinical and microbiological parameters and prostaglandin E_2 levels in gingival crevicular fluid. *J Periodontol* 2006;**77**: 437–43.
34. Airila-Mansson S, Soder B, Kari K, Meurman JH. Influence of combinations of bacteria on the levels of prostaglandin E_2, interleukin-1beta, and granulocyte elastase in gingival crevicular fluid and on the severity of periodontal disease. *J Periodontol* 2006;**77**:1025–31.
35. Vardar S, Buduneli E, Baylas H, Berdeli AH, Buduneli N, Atilla G. Individual and combined effects of selective cyclooxygenase-2 inhibitor and omega-3 fatty acid on endotoxin-induced periodontitis in rats. *J Periodontol* 2005;**76**:99–106.
36. Shimamoto Ch, Fujiwara S, Kato M, Ito S, Katsu K, Mori H, et al. Inhibition of Ach-stimulated exocytosis by NSAIDs in guinea pig antral mucous cells: autocrine regulation of mucin secretion by PGE_2. *Am J Physiol Gastrointest Liver Physiol* 2005;**288**:39–47.