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ORIGINAL ARTICLE

Increase nitric oxide synthase activity in parotid glands from rats with experimental periodontitis

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OBJECTIVE: In this study we investigated the activity of the nitric oxide synthase (NOS) in parotid glands from rats with experimental periodontitis and controls.
METHODS: Periodontitis was produced by a ligature placed around the cervix of the two lower first molar. Experiments were carried out 22 days after the ligature.
RESULTS: Ligation caused an increase in parotid NOS activity. The selective blocker of the inducible isoform of the enzyme partially inhibited its activity in parotid glands from rat with ligature. In controls, the activity was partially inhibited by the antagonists of the selective neural and endothelial isoforms. NOS activity in rats with ligature was cyclic adenosine monophosphate (cAMP)-dependent while in controls it was calcium-dependent. Prostaglandin E_2 concentration was increased in parotid gland from rats with ligature. The inhibitor of prostaglandin production, FR 122047, diminished both, prostaglandin production and NOS activity. In rats with ligature unstimulated amylase released is increased. Both, prostaglandin and NOS were involved in the increment of amylase release.
CONCLUSION: It can be concluded that in parotid glands from ligated rats, prostaglandin E_2 production is increased and, through cAMP accumulation, activates the inducible NOS isoform. The increment of nitric oxide production participates in the increase in basal amylase release.
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Keywords: nitric oxide synthase; parotid gland; experimental periodontitis; prostaglandins; amylase

Introduction

Salivary glands may respond to periodontitis by enhanced synthesis and secretion of some acinar proteins. Cystatin C and amylase are present in almost twofold higher concentrations in parotid saliva of periodontitis patients (Henskens *et al*, 1996) On the other hand, glandular saliva from juvenile periodontitis subjects shows higher concentrations of epidermal growth factor (Hormia *et al*, 1993). The specific chitinase activity, from patients with periodontitis, is significantly increased in glandular saliva of parotid, palatine, submandibular and sublingual glands (Van Steijn *et al*, 1999). In addition, immunoglobulin A output increases in salivary glands in response to human experimental gingivitis (S\eeman *et al*, 2004) These results provide evidence that salivary glands may respond to oral disease by enhanced synthesis and secretion of salivary proteins, some of which increase the protective potential of saliva. *In vitro* studies, which used a well established rat model of periodontitis (Lohinai *et al*,1998), showed an increase in mucin and amylase output in submandibular and parotid glands respectively (Busch *et al*, 2008; Miozza *et al*, 2009).

In recent years, there has been an increasing interest in the role of nitric oxide (NO) in the pathogenesis of oral and periodontal diseases (Uğar-Çankal and Ozmeric, 2006). NO plays an important role as a regulator of the salivary gland functions in physiological and pathological conditions (Ohashi *et al*, 1999). It has a dual behavior in the control of salivary gland functions. It can act as a physiological messenger of several neurotransmitter receptors (Moncada *et al*, 1991) or as an inflammatory mediator in a growing variety of diseases (Nathan, 1997). In this regard, it has been documented that the NO signaling pathway is involved in salivary secretion (Edwards and Garrett, 1993; Lomniczi *et al*, 1998; Lohinai *et al*, 1999). On the other hand, in the submandibular gland of non-obese diabetic mouse nitric oxide synthase (NOS) activity presents a marked decrease and a differential expression of endothelial NOS (eNOS) was observed (Rosignoli *et al*, 2001). NO in periodontal tissue and saliva may be part of the non-specific natural defense mechanisms of

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the oral cavity against pathogenic bacteria. But alternatively, excessive amounts of NO may contribute to tissue destruction in periodontitis (Lohinai *et al*, 1998). Elevated production of salivary NO was detected in patients with oral mucosal diseases containing oral lichen planus and aphtous ulceration. These facts suggest that excessive salivary NO played a potential role in modifying oral mucosal diseases as a physiopathological regulator (Ohashi *et al*, 1999).

Salivary glands may respond to periodontitis by modifying the synthesis and secretion of some proteins. NO has a role on salivary gland functions in physiological and pathological conditions. Thus, we hypothesized that NO production could be increased in parotid glands from periodontitis rats. The main purpose of this study was to investigate the activity of NOS in parotid glands during ligature-induced periodontitis in rats and its relation with amylase release.

Materials and methods

Animals

Male Wistar rats weighing 250–300 g were lightly anesthetized with a mixture of Ketamine and Xilazine (50 and 5 mg k^{-1} respectively). A black thread was placed around the cervix of the two lower first molars and knotted mesially. '*In vitro* studies' were performed in pieces of parotid gland tissue. Assays 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).

Nitric oxide synthase activity

Nitric oxide synthase activity was measured in parotid glands using L-[U-^{14}C]arginine as substrate (Bredt and Snyder, 1990). Briefly, 50–60 mg of gland tissue was incubated with 0.4 μCi L-[U-^{14}C]arginine (Amersham Pharmacia Biotech, Buckinghamshire, England, about 300 mCi $mmol^{-1}$) in 500 μl of Krebs Ringer bicarbonate solution (KRB: NaCl 137, KCl 5, $CaCl_2$ 4, $MgSO_4$ 1, $NaHPO_4$ 1, $NaHCO_3$ 24 and glucose 11, mM), pH 7.4 gassed with 5% CO2 in O2 for 30 min at 37°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, Life Science Research, Hercules, CA, USA). When used, inhibitors of NOS activity L-*N*-monomethylarginine (L-NMMA, non-selective NOS inhibitor) (Sigma Chemical Co., St Louis, MO, USA), L-Nio-dihydrochloride (Nio, potent inhibitor of eNOS III isoform), 3-Bromo-7-nitroindazole (Nz, potent inhibitor of neural NOS I isoform) and aminoguanidine [AMG, irreversible inhibitor of inducible NOS (iNOS) II isoform] (Tocris Cookson Inc., Ellisville, MO, USA) or inhibitors of prostaglandin production, indomethacin (non-selective COX inhibitor), FR 122047 (selective COX-1 inhibitor) and DuP 697 (potent and selective COX-2 inhibitor) (Tocris Cookson Inc.), were included from the beginning of the incubation time. The activity of constitutive isoforms was assessed by incubation the tissues in KRB without calcium and with 5 mM EGTA. NO production (measured as pmol of [^{14}C]citrulline) was expressed as picomol of [^{14}C]citrulline per gram of tissue wet weight (pmol g^{-1} wet wt).

Determination of prostaglandin production

Parotid glands (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 37°C. When used, the cyclooxygenase (COX) inhibitors indomethacin (non-selective) (Sigma Chemical Co.), FR 122047 (selective COX-1 inhibitor) and DuP 697 (selective COX-2 inhibitor) (Tocris Cookson Inc.) were included from the beginning of the incubation time. After homogenization, all procedures employed were those indicated in the protocol of Prostaglandin E2 Biotrak Enzyme Immuno Assay (ELISA) System (Amersham Biosciences, Piscataway, NJ, USA). The results are expressed as picogram of PGE2 per milligram of tissue wet weight (pg mg^{-1} wet wt).

Experimental procedure for amylase assay

Parotid glands (15 mg) were incubated in 500 μl of KRB solution pH 7.4 without glucose and with 5 mM β-hydroxybutyric acid, bubbled with 95% O_2 and 5% CO_2 at 37°C for 30 min. Inhibitors were included from the beginning of the incubation time. Amylase activity was determined by the method described by Bernfeld (1955) using starch suspension as the substrate. Amylase activity is expressed in terms of U mg^{-1} wet weight where 1 U amylase was defined as the quantity of enzyme that liberates 1 mg of maltose for 1 min at 20°C.

Statistical analysis

Statistical significance of differences was determined by the Newman–Keuls test after analysis of variance, while two-sample comparisons were made using Student's *t*-tests. Differences between means were considered significant at $P < 0.05$.

Results

Figure 1 shows the activity of NOS, expressed as pmol g^{-1} wet weight, in parotid glands from rats with experimental periodontitis and controls. A significant increase in NOS activity could be seen in parotid glands from periodontitis rats. The activation of NOS was inhibited by 56% and 67% in control and periodontitis group respectively, by 5×10^{-5} M L-NMMA (Figure 1). To further explore whether this marked increase in NOS activity in parotid glands was resulting from constitutive or inducible NOS isoforms, we carried out the assays in the presence of Nio and Nz inhibitors of the constitutive isoforms endothelial (eNOS) and neuronal (nNOS) respectively and AMG, inhibitor of the iNOS isoform. As shown in Figure 2, in control group NOS activity was inhibited by 50% by Nz and in 27% by Nio, indicating that both isoforms participate in total NOS

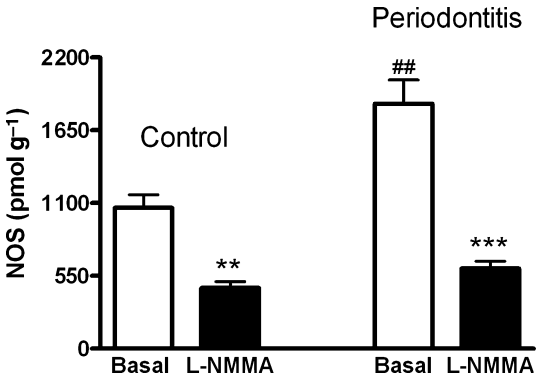

Figure 1 NOS activity in parotid glands from rats with experimental periodontitis and controls. Parotid glands were incubated with L-[U-^{14}C] arginine and the activity of NOS was determined as described under Materials and methods. Assays were carried out in the absence (basal) and presence of 5×10^{-5} M L-N^{G}-monomethylarginine (L-NMMA) as indicated in the graph. Bars represent the mean ± s.e.m. of six experiments. ##Significantly different from basal control ($P < 0.01$); **significantly different from basal control ($P < 0.01$); ***significantly different from basal periodontitis ($P < 0.001$)

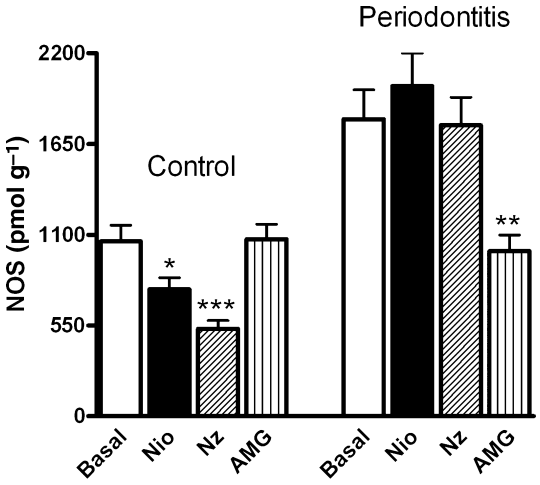

Figure 2 NOS activity in parotid glands from rats with experimental periodontitis and controls. Parotid glands were incubated with L-[U-^{14}C] arginine and the activity of NOS was determined as described under Materials and methods. Assays were carried out in the absence (basal) and presence of 5×10^{-6} M L-Nio-dihydrochloride (Nio), or 3-Bromo-7-nitroindazole (Nz) or aminoguanidine (AMG) as indicated in the graph. Bars represent the mean ± s.e.m. of six experiments. *Significantly different from basal control ($P < 0.05$); **significantly different from basal periodontitis ($P < 0.01$); ***significantly different from basal control ($P < 0.001$)

activity, being the nNOS the predominant. By contrast, NOS activity in parotid glands from rats with experimental periodontitis was only inhibited in the presence of AMG indicating the presence of the iNOS isoform in this group (Figure 2). The inhibitors of the constitutive NOS isoforms failed in inhibiting NOS activity in periodontitis group. This fact was probably attributable to the changes in the ratio of constitutive/inducible NOS isoform, which involved a down-regulation of the constitutive NOS and an up-regulation of the iNOS as was described in other tissues. (Palatka *et al*, 2006).

Table 1 Activity of the nitric oxide synthase in parotid glands from rats with experimental periodontitis and controls

Groups	*Control*	*Periodontitis*
Basal	1070 ± 98	1750 ± 120[a]
EGTA	750 ± 60[b]	1500 ± 130
SQ 22536	1068 ± 89	1120 ± 100[c]

Parotid glands from rats with experimental periodontitis and controls were incubated with L-[U-^{14}C]arginine and the activity of NOS (pmol g^{-1} wet weight) was determined as described in Materials and methods. Assays were carried out in the absence (basal) and presence of 5×10^{-3} M EGTA or 5×10^{-6} M SQ 22536 as indicated in the table. Values represent the mean ± s.e.m. of six experiments.
[a]Significantly different from basal control, $P < 0.01$.
[b]Significantly different from basal control, $P < 0.05$.
[c]Significantly different from basal periodontitis $P < 0.01$.

Constitutive NOS isoforms are calcium-dependent (Nathan and Xie, 1994) while iNOS has been related to cAMP–protein kinase A (PKA) system (Onga *et al*, 2000; Farghali *et al*, 2008). To evaluate the role of calcium and cAMP in NOS activity, we incubated the glands from rats with ligature and controls with EGTA and the inhibitor of the adenylyl cyclase, SQ 22536 (Sigma Chemical Co.), and then determined NOS activity. As shown in Table 1, in control group the predominant isoforms active seems to be the constitutive as derived from EGTA assays. Conversely, in periodontitis group NOS activity was inhibited in the presence of the inhibitor of adenylyl cyclase, SQ 22536, 5×10^{-6} M.

Inducible NOS is expressed during inflammation thus, to see whether the increase basal activity of NOS observed in parotid gland from experimental periodontitis rats was related to prostaglandin production, we incubated the glands with the non-selective COX inhibitor, indomethacin (5×10^{-6} M), the COX-1 selective inhibitor, FR 122047 (5×10^{-8} M), and the COX-2 selective inhibitor, DuP 697 (1×10^{-8} M). As shown in Figure 3, NOS activity in parotid glands from

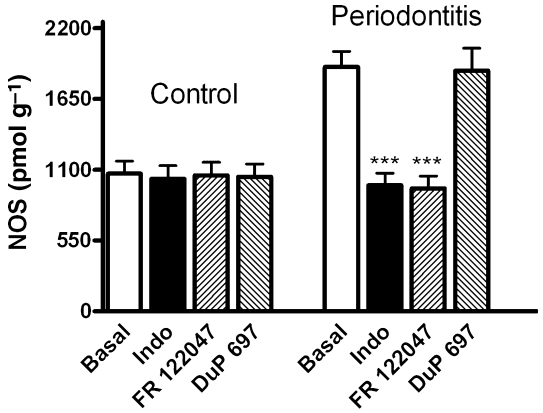

Figure 3 NOS activity in parotid glands from rats with experimental periodontitis and controls. Parotid glands were incubated with L-[U-^{14}C] arginine and the activity of NOS was determined as described under Materials and methods. Assays were carried out in the absence (basal) and presence of 5×10^{-6} M indomethacin, or 5×10^{-8} M Fr 122047, or 1×10^{-8} M DuP 697 as indicated in the graph. Each bar represents the mean ± s.e.m. of six experiments. ***Significantly different from basal periodontitis ($P < 0.001$)

Table 2 Prostaglandin E_2 accumulation in parotid glands from rats with experimental periodontitis and controls

Groups	*Control*	*Periodontitis*
Basal	23 ± 2.0	34 ± 3.0[a]
Indomethacin	13 ± 1.2[b]	18 ± 1.6[b]
FR 122047	11 ± 1.4[b]	20 ± 2.0[b]
DuP 697	22 ± 2.1	28 ± 2.6

Parotid glands from rats with experimental periodontitis and controls were incubated during 30 min and the concentration of PGE_2 (pg mg^{-1} wet weight) was determined as described in Materials and methods. Assays were carried out in the absence (basal) and presence of 5×10^{-6} M indomethacin, 5×10^{-8} M FR 122047 or 1×10^{-8} M DuP as indicated in the table. Values represent the mean ± s.e.m. of four experiments.
[a]Significantly different from basal control, $P < 0.05$.
[b]Significantly different from basal of the corresponding group, $P < 0.01$.

periodontitis rats was decreased in the presence of indomethacin and FR 122047 (Figure 3).

As prostaglandin seemed to be involved in NOS activity, we evaluated its concentration in parotid glands from rats with experimental periodontitis and controls. As shown in Table 2, prostaglandin production was increased in parotid glands from rats with experimental periodontitis. Basal values of both groups were diminished in the presence of the non-selective COX inhibitor indomethacin and the selective COX-1 inhibitor FR 122047 but not in the presence of the selective COX-2 inhibitor DuP 697 (Table 2).

To investigate the relation of NOS activity with the current secretory function of parotid gland, we determined amylase release under basal conditions. Figure 4 shows that periodontitis rats presented a significant increase in basal amylase release as described previously (Miozza *et al*, 2009) The increment was inhibited in the presence of either an NOS inhibitor (L-NMMA 5×10^{-5} M) or a prostaglandin production inhibitor (indomethacin 5×10^{-6} M).

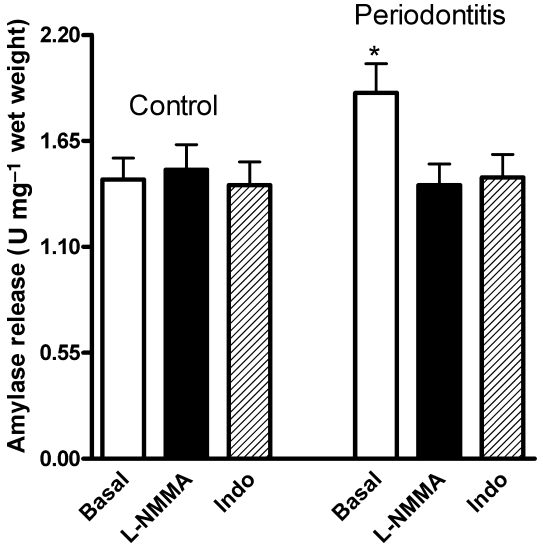

Figure 4 Unstimulated amylase release in parotid glands from rats with experimental periodontitis and controls. Parotid glands were incubated in the absence (basal) and presence of 5×10^{-5} M L-NMMA or 5×10^{-6} M indomethacin and the amylase activity in the medium was determined as stated in Materials and methods. Bars represent the mean ± s.e.m. of six experiments. *Significantly different from all other groups ($P < 0.05$)

Discussion

Salivary glands may respond to periodontitis by enhanced synthesis and secretion of some proteins. Because NO plays an important role as a regulator of the salivary gland secretory functions in physiological and pathological conditions and salivary NO is elevated in patients with oral mucosal disease (Ohashi *et al*, 1999), we hypothesized that NO production could be increased in salivary glands from subjects with periodontitis. In this study, a well established rat model of periodontitis which involves a ligature around the cervix of the two mandibular first molar teeth (Lohinai *et al*, 1998), was utilized for studying the *in vitro* activity of the NOS in parotid gland from rats with ligature.

Nitric oxide is a free radical that plays a role in the host defensive response to infection in oral tissues and is synthesized from the conversion of L-arginine to L-citruline by NOS. NOS exist in three distinct isoforms; neuronal NOS (nNOS), eNOS and iNOS. While eNOS and nNOS are constitutively expressed and release small amounts of NO, iNOS is expressed in response to proinflammatory stimuli and produces large amount of NO (Förstermann *et al*, 1994; Lyons, 1995).

Our results showed an increase in NOS activity in parotid glands after 22 days of ligature. To further explorer whether any difference in the activation of the three isoforms of NOS was present, in parotid glands from periodontitis rats, we studied the effect of the selective inhibitors of NOS activity. The results argue for differential activity of iNOS as there was clear difference in the effect of the selective NOS inhibitors. NOS activity of control group was inhibited by the selective inhibitors of the neuronal and endothelial isoforms while NOS activity from rats with ligature was only inhibited by AMG, selective inhibitor of the iNOS.

The enzymes eNOS and nNOS are regulated by Ca^{++}/calmodulin, contrary to iNOS which is activated by cytokines and bacterial products. On the other hand, *in vitro* studies showed that the iNOS may be activated in response to physiological stimulus such us hypertension and glucose homeostasis or pathological such as inflammation, via cAMP/PKA pathway (Onga *et al*, 2000; Tai *et al*, 2007; Farghali *et al*, 2008). In our study, NOS activity in control group was calcium-dependent while NOS activity in periodontitis group was calcium independent and cAMP-dependent. In addition, this result indicates that in parotid glands from rats with ligature the predominant NOS isoform active was the inducible as derived from EGTA and SQ 22536 assays.

Prostaglandin production was increased in parotid glands from rats with experimental periodontitis. An intimate contact of plaque covered molar surfaces can enable production of inflammatory mediators such as

PGE_2, one of the major metabolites of COX, in parotid glands (Ohshima *et al*, 1987). Prostaglandins induce cAMP accumulation through the activation of EP2 receptor (Narumiya *et al*, 1999) and *in vitro* studies showed an increase of cAMP accumulation in parotid glands from rats with experimental periodontitis (Miozza *et al*, 2009).

On the basis our results, we can hypothesized that in parotid glands from rats with ligature, the increase of NOS activity may be attributable to PGE_2 production, which acting through its own receptor in parotid acinar cells, induce an increase in cAMP. This increase in cAMP levels results in the activation of iNOS. Interestingly, the augmentation of NO synthesis by PGE_2 in inflammatory process through cAMP–PKA system has been described, *in vitro*, in other tissue (Onga *et al*, 2000).

The secretion of amylase from the rat parotid gland induced by the stimulation of β-adrenergic and vasoactive intestinal peptide receptors involve the activation of NO/cGMP (cyclic guanosine monophosphate) signaling pathway (Sayardoust and Ekström, 2003). In addition, prostaglandins have a role in modulating amylase release in parotid glands (Hata *et al*, 1990). Basal amylase release is increased in parotid glands from rats with ligature (Miozza *et al*, 2009). Thus, we investigated the participation of NO in amylase release in parotid glands from rats with experimental periodontitis and controls. As expected, the inhibition of NO and PG returned amylase basal values in ligated rats comparable with that of control ones. Although NO has been described of little importance for the basal amylase release in parotid glands (Sayardoust and Ekström, 2003), the increment of its production observed in parotid glands from rats with periodontitis might contribute to the increase in amylase release in unstimulated conditions.

The observations described above show that parotid glands from ligated rats present an increase in NOS activity, and this can be explained by the increased production of PGE_2 in the gland which in turn activates the inducible isoform of NOS. The increment of NO production participates in the increase in basal amylase release.

Our results contribute to establish a role of NO and PGE in salivary secretion in physiological and pathological conditions as described previously in rats (Lomniczi *et al*, 2001).

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Author contributions

Valeria Miozza did the experiments and contribute to the analysis and interpretation of the data. Enri Borda and Leonor Sterin-Borda revised the manuscript and approved the version to be published. Lucila Busch contributed with the conception and design of the experiments, the analysis and interpretation of the data, drafting the manuscript and approval the final version to be published.

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