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Nitric oxide synthase I and VIP-activated signaling are affected in salivary glands of NOD mice

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Abstract

The autoimmune sialadenitis developed by non-obese diabetic (NOD) mice is considered a suitable model to study the ethiopathogenic mechanisms leading to *sicca symptoms* in Sjögren's syndrome (SS). Evidence supporting a neural rather than immune origin of the secretory dysfunction has been provided. As both nitric oxide and vasoactive intestinal peptide (VIP) are common messengers to nervous and immune systems mediating secretory and inflammatory responses, we examined nitric oxide synthase (NOS) activity with special focus on VIP-mediated effects in salivary glands of NOD mice. We found a decreased NOS activity and expression in major salivary glands of NOD mice with respect to control mice. In addition, there was a deficient VIP-activated signaling associated with a reduced saliva and amylase secretion in response to VIP. Our results support the hypothesis of an impaired balance of neuroimmune interactions in salivary glands as early events to take place in the progressive loss of secretory function of NOD mice.

Keywords: Nitric oxide; NOS isoforms; NOD mice; VIP signaling; Salivary glands; Autoimmune sialadenitis

1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune rheumatic disease characterized by a severe dryness of the mouth and the eyes (Strand and Talal, 1980; Jacobsson and Manthorpe, 1995; Fox and Michelson, 2000). The poor correlation between the presence of lymphomononuclear infiltration in salivary glands and the marked impairment of salivary and lacrimal secretion has prompted the investigators to suggest that neural rather than immune regulatory pathways may have a role in the pathogenesis of *sicca syndrome* (Fox and Michelson, 2000). The ethiopathogenic mechanisms leading to SS are still unclear and multiple approaches have been proposed to further understand them. In this regard, the hypothesis of an impaired balance of normally occurring neuroimmune interactions in the target organ as the earliest events to take place in the overall exocrinopathy seems attractive and several studies on SS patients and on experimental models can be analyzed in line with this approach. Particularly, the availability of various mouse models of sialadenitis offers the unique possibility of studying such interactions in major salivary glands. Among them, the *non-obese diabetic* (NOD) mouse is considered a suitable model of SS associated with other connective tissue diseases (secondary SS) (Miyagawa, 1986; Humphreys-Beher et al., 1994; Yamano et al., 1999). NOD mice have the advantage over other SS models of developing a deep loss of secretory function along with the presence of lymphomononuclear infiltrates in exocrine tissues (Hu et al., 1992; Humphreys-Beher et al., 1994). Interestingly, as it was reported for SS patients, evidence supporting a non-immune origin for the secretory dysfunction in this model has been provided: Certainly, congenic immunodeficient NOD mice lack the ability to produce functional B- and T-lymphocytes and nonetheless they showed a loss of glandular function associated with an increased programmed cell death (Kong et al., 1998) and, in NOD mice, lymphocyte infiltration of parotid glands poorly correlated with saliva secretion in agreement with observations reported in SS patients (Yamamoto et al., 1996). Consistent with this, we have recently reported on alterations in the activity and expression of nitric

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oxide synthase (NOS) in submandibular glands of NOD mice at an early stage of disease (Rosignoli et al., 2001). The effect involved only one isoform of the enzyme and was not observed in non-exocrine tissues. Moreover, it is well known that parasympathetic stimulation leading to watery saliva involves acetylcholine and vasoactive intestinal peptide (VIP) receptors (Ekström et al., 1982, 1983; Lundberg et al., 1982) and that both neurotransmitters activate nitric oxide signaling (Perez Leiros et al., 2000; Murthy et al., 1993). As predicted from these assumptions, NOS alterations in submandibular glands of NOD mice were associated to a diminished secretory response to VIP (Rosignoli et al., 2001). It is worth noting that nitric oxide and VIP are common messengers to both nervous and immune systems. Certainly, in addition to their role in the nervous regulation of secretion, nitric oxide is a prominent inflammatory mediator in various rheumatic diseases (Amin et al., 1999), whereas VIP was shown to modulate inflammatory responses through its action on macrophages (Pozo et al., 2000). In an attempt to further characterize the alterations in NOS activity and the effect of VIP on salivary glands of NOD mice in association with the progressive loss of secretory function, we investigated the activity and expression of nitric oxide synthase in submandibular and parotid glands from prediabetic NOD mice with special focus on VIP-mediated effects. Here we report that NOS activity and expression are affected to similar extents in both major salivary glands of NOD mice regardless of the sex studied. Also, we present evidence to indicate a deficient VIP-mediated signaling in parotid and submandibular glands of NOD mice associated with a reduced saliva, protein and amylase secretion in response to VIP.

2. Materials and methods

2.1. Animals

NOD and BALB/c mice (16–19 weeks old unless otherwise stated) were bred and maintained under pathogen free conditions in the Central Animal Care facility at the University of Buenos Aires (Ciudad Universitaria, Buenos Aires). NOD mice were routinely tested for blood glucose levels using the glucose oxidase method in 20 μl samples of NOD and control sera (Wiener lab, Rosario, Argentina). NOD mice used were considered pre-diabetic as their values of glucose (130±13 mg/dl, n=5) did not significantly differ from those of control mice (113±9, n=5). Salivary glands from NOD mice used throughout showed no signs of histological damage or mononuclear cell infiltration as observed on slices from glands embedded in paraffin and stained with haematoxylin-eosin and reported previously (Perez Leiros et al., 1994). All studies were conducted according to standard protocols of the Animal Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, University of Buenos Aires.

2.2. Salivary flow rates and amylase secretion

Total saliva was collected after stimulation of secretion with either the muscarinic agonist pilocarpine (Sigma, MO, USA) (50 μg pilocarpine/100 g weight) injected intraperitoneally in mice fasted overnight or pilocarpine plus VIP (Sigma) (10 μg VIP/100 g weight). By means of a micropipette, saliva accumulated in the oral cavity was driven to microtubes in ice for 12 min following injection. The flow rate was calculated as the volume of saliva collected, measured by weighing tubes before and after collection, in microliters per minute and per 100 g of body weight. The concentration of protein and amylase in saliva was determined in an aliquot of the fluid collected from each mouse by the methods of Lowry et al. (1951) and Bernfeld (1955), respectively. The results were expressed in μg protein/μl of saliva collected per minute per 100 g body weight or amylase units/ml of saliva/min/100 g body weight. One unit of amylase was defined as the activity of amylase that released 1 mg of maltose per min at 20 °C (Bernfeld, 1955).

2.3. Nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in submandibular and parotid glands from fasted mice using L-[U-^{14}C]-arginine as substrate as described earlier (Perez Leiros et al., 2000; Rosignoli and Pérez Leirós, 2002). Whole glands were incubated with 0.2 μCi L-[U-^{14}C]-arginine (Amersham Pharmacia Biotech, Buckinghamshire, England, about 300 mCi/mmol) in 500 μl of Krebs–Ringer-bicarbonate (KRB) solution pH 7.4 gassed with 5% CO_2 in O_2 at 37 °C for 30 min except for time-course studies where the incubation was stopped at different times. Specific NOS activity was calculated as total activity minus that measured in the presence of 50 μM L-N^G-mono-methyl arginine (L-NMMA) (Sigma) and the activity of constitutive isoforms (calcium-dependent) was assessed by incubating the tissues in KRB without calcium and with 5 mM EGTA. When used, inhibitors were included from the beginning of the incubation time and VIP (Sigma) was added at the final concentrations indicated for the last 15 min.

2.4. Immunoblotting

Submandibular and parotid glands were excised from fasted mice and immediately homogenized at 4 °C in 50 mM Tris–HCl buffer pH 7.5 with 0.15% Triton X-100 and protease inhibitors as previously reported (Rosignoli et al., 2001). Once centrifuged at 5000×g 10 min at 4 °C, supernatants were frozen at −80 °C until used and an aliquot of each sample was separated for protein determination. Extracts (100 μg protein/lane), positive controls for each isoform of NOS (Transduction Labs, KY, USA) and molecular weight standards (Amersham Pharmacia Biotech, NJ, USA) were subjected to 7.5% SDS-PAGE, transferred to nitrocellulose membranes and revealed with specific

monoclonal antibodies against NOS I and III (Transduction Labs) using an alkaline phosphatase conjugate (Sigma) as we have described earlier (Rosignoli et al., 2001).

2.5. cAMP determination

We determined adenosine 3′,5′-cyclic monophosphate (cAMP) levels in submandibular and parotid glands by means of an enzyme immunoassay kit from Assay Designs (MI, USA). Samples were prepared by incubating the tissues for 30 min in 1 ml KRB with 5% CO_2 in O_2 and VIP or isoproterenol were added in the last 10 min at the final concentrations indicated. Submandibular and parotid glands were then homogenized and cAMP was extracted in ethanol for subsequent determination. cAMP was measured according to the manufacturers and results were expressed in pmol/mg tissue wet weight.

2.6. Statistical analysis

Statistical significance of differences was determined by the two-tailed *t*-test for independent populations. When multiple comparisons were necessary, the Student–Newman–Keuls test was used after analysis of variance. Differences between means were considered significant at $P<0.05$.

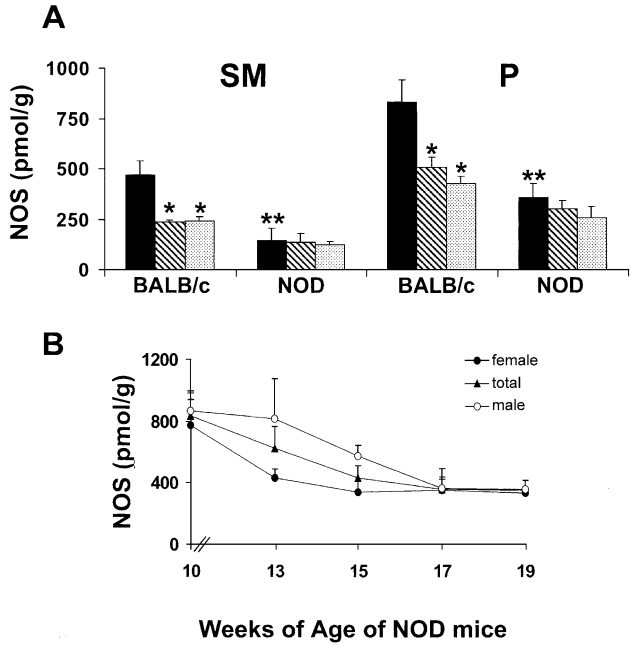

Fig. 1. NOS activity in submandibular and parotid glands of NOD and control mice. (A) Submandibular (SM) and parotid (P) glands of 16–19 weeks old NOD and BALB/c mice were incubated with L-(U-^{14}C)-arginine and the activity of NOS was determined as described in Materials and methods. Assays were carried out in the absence (■) and presence of 50 μM L-N^G-mono-methyl arginine (▨) or 5 mM EGTA (▩) as indicated in the graph. Values represent the mean±S.E.M. of at least five experiments. *Significantly different from basal BALB/c without additions, $P<0.05$; **significantly different from basal BALB/c without additions, $P<0.01$. (B) Parotid glands from NOD mice of 10 and 13–19 weeks of age were used for NOS assays as indicated above. Results shown are the mean±S.E.M. of at least four determinations.

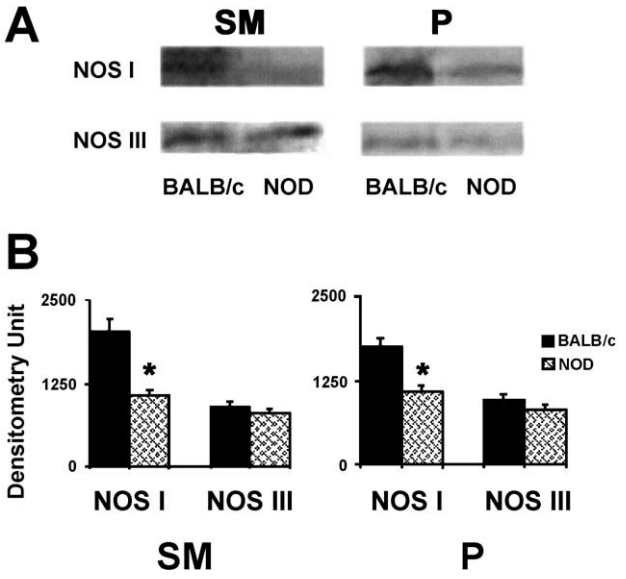

Fig. 2. Expression of NOS isoforms in submandibular and parotid glands of NOD mice. (A) Tissue extracts from control BALB/c mice or from NOD mice were obtained as described in Materials and methods. Extracts from submandibular glands (SM) and parotid glands (P) (100 μg protein/lane) were subjected to 7.5% SDS-PAGE and then to immunoblotting assays with specific monoclonal antibodies against NOS I and NOS III as pointed out in the figure. The relative molecular weight for NOS I was 155 kDa and for NOS III, 140 kDa. Results shown are representative of at least 4 similar experiments. (B) Densitometric analysis was expressed in arbitrary densitometric units corresponding to the mean±S.E.M. of at least four independent experiments. *$P<0.05$ compared to BALB/c.

3. Results

3.1. Nitric oxide synthase activity and expression in salivary glands of NOD mice

Fig. 1A shows the complete loss of specific nitric oxide synthase activity in submandibular and parotid glands of 16–19 weeks old NOD mice compared to control BALB/c

Table 1
Effect of VIP on nitric oxide synthase activity in salivary glands of NOD mice

NOS (pmol/g)	Submandibular glands		Parotid glands	
	BALB/c	NOD	BALB/c	NOD
Basal	457±41	158±39	832±96	360±69
VIP (10^{-8} M)	694±89[a]	199±82	1477±153[a]	440±86

Submandibular and parotid glands of 16–19 weeks old NOD and BALB/c mice were incubated with L-(U-^{14}C)-arginine and the activity of NOS was determined as described in Materials and methods. Assays were carried in the absence (basal) and presence of VIP as indicated. Values represent the mean±S.E.M. of at least four experiments.

[a] Significantly different from basal values, $P<0.05$.

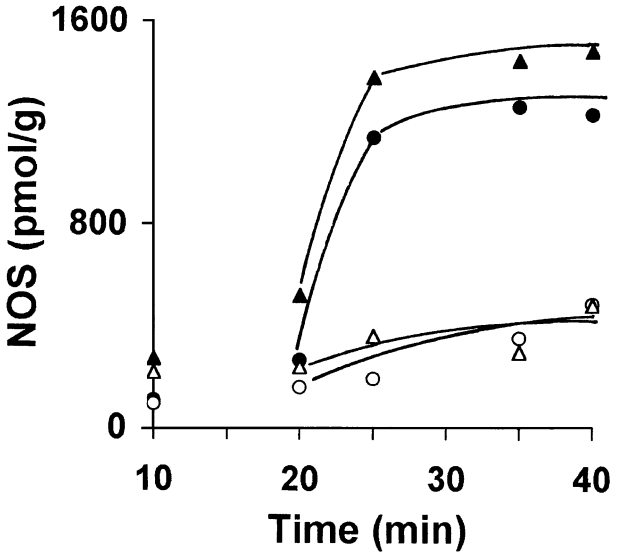

Fig. 3. Time-course of activation for NOS in salivary glands of NOD and control mice. Salivary glands of 18 weeks old mice were incubated with L-(U-^{14}C)-arginine and the activity of NOS was determined as described in Materials and methods at the times indicated in the graph. BALB/c submandibular (●—●) and parotid (▲—▲) glands; NOD submandibular (○—○) and parotid glands (△—△). Shown is an experiment representative of four other separate experiments with similar results.

mice. The isoforms involved in the effect seemed to be constitutive isoforms since there were no significant differences between NOS activity measured in the presence of EGTA and LNMMA in salivary glands from both groups of mice. Fig. 1A also shows that NOS is affected to similar extents in parotid and submandibular glands as we have reported previously for younger NOD mice (Rosignoli et al., 2001). Moreover, as it can be seen in Fig. 1B, there was a delay of about 2 weeks in the appearance of NOS alterations in parotid glands from NOD males compared to females and the progression of the effect was parallel in males and females thereafter, reaching a complete lack of NOS activity at 16–19 weeks of age. Similar results were obtained in submandibular glands (data not shown).

In order to explore whether the loss of specific NOS activity in 16–19 weeks old NOD mice was associated to an altered expression of the enzyme and based on the observation that only constitutive NOS isoforms seemed to be involved, we carried out immunoblotting experiments in salivary glands of NOD and control mice with monoclonal antibodies against NOS I and NOS III. Fig. 2 shows that only NOS I expression was affected in both parotid and submandibular glands, whereas no changes were observed in the expression of NOS III.

3.2. Effect of VIP on signaling and secretion in salivary glands of NOD mice

On the basis that VIP is known to potentiate muscarinic receptor-mediated secretion in salivary glands, that VIP activates nitric oxide signaling and that nitric oxide is currently associated with an increased saliva flow, first, we investigated the effect of VIP on NOS activity in salivary glands of NOD mice. Table 1 shows that VIP failed to stimulate NOS activity in both submandibular and parotid glands of NOD mice as it did in control BALB/c mice. In order to further investigate if the decreased responsiveness of the NOS was associated with differences between BALB/c and NOD mice enzyme kinetic curves, we studied the time-course of activation for NOS. Fig. 3 shows that NOS activation measurement was optimal at 30–40 min in both glands from control mice, whereas NOD mice showed no detectable specific activity of NOS at any time

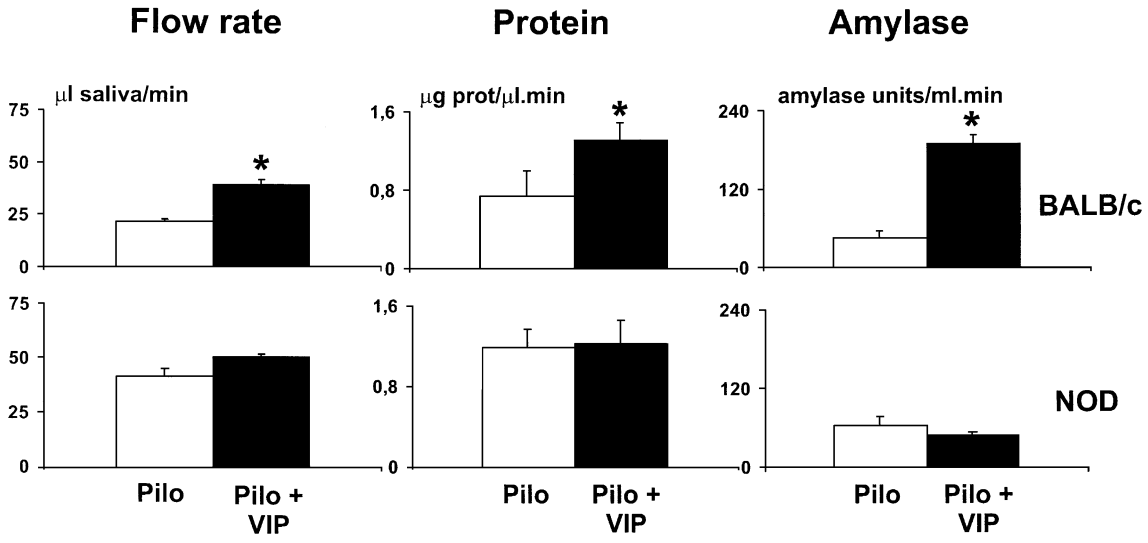

Fig. 4. Effect of VIP on salivary flow rate, protein and amylase secretion of NOD mice. NOD and BALB/c mice fasted overnight were injected with pilocarpine (Pilo) or a mixture of pilocarpine and VIP (Pilo+VIP) and saliva was collected during 12 min following injection as indicated in Materials and methods. Results shown are the mean±S.E.M. of at least five experiments. *Significantly different from Pilo in BALB/c mice, $P<0.05$.

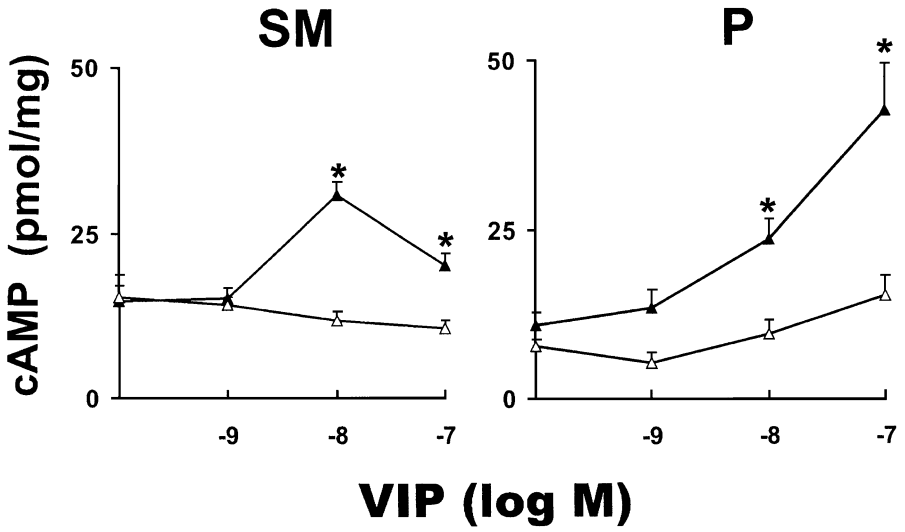

Fig. 5. cAMP levels in response to VIP in salivary glands of NOD mice. Submandibular (SM) and parotid (P) glands of NOD (△—△) and BALB/c (▲—▲) mice fasted overnight were incubated with increasing concentrations of VIP for cAMP determination as described in Materials and methods. Results shown are the mean±S.E.M. of at least four experiments. *Significantly different from basal values in the absence of VIP, $P<0.05$.

tested. Moreover, extended incubation times in the presence of VIP (20 to 40 min) in either BALB/c or NOD glands did not stimulate NOS activity beyond the values obtained at 15 min presented in Table 1 (data not shown). Regarding the secretory effect of VIP on salivary glands of NOD mice, Fig. 4 shows that in vivo treatment of NOD mice with VIP did not induce a potentiation of muscarinic-induced saliva flow rate compared to BALB/c controls. Moreover, as it can be seen in Fig. 4, VIP also failed to stimulate protein and amylase secretion in NOD mice to the same levels observed in control mice. As proteinaceous secretion by salivary glands is linked to cAMP signaling pathways, we investigated whether the reduced effect of VIP on protein secretion in NOD mice was due to a deficient production of the cyclic nucleotide in the glands. Fig. 5 shows the concentration-dependent stimulation of cAMP levels in both parotid and submandibular glands from control BALB/c mice. In contrast, there was no significant effect of VIP in submandibular glands and parotid glands of NOD mice. Moreover, this reduced response to VIP observed in NOD salivary glands seemed not to be due to an abnormal coupling of Gs protein to the effector adenylyl cyclase since isoproterenol, by using the same transduction pathway, was equally effective to stimulate cAMP levels in either NOD or BALB/c submandibular and parotid glands (Table 2).

Table 2
Effect of isoproterenol on cAMP levels in salivary glands of NOD mice

cAMP (pmol/mg)	Submandibular glands		Parotid glands	
	BALB/c	NOD	BALB/c	NOD
Basal	14.8±2.4	15.4±3.3	10.8±2.1	7.8±1.0
iso (10^{-5} M)	181.3±26.4[a]	169.8±25.5[a]	94.1±20.1[a]	57.8±10.9[a]

Salivary glands of 16–19 weeks old NOD and BALB/c mice were incubated in the absence (basal) or presence of isoproterenol (iso) to determine cAMP levels. Values represent the mean±S.E.M. of at least four experiments.

[a] Significantly different from basal values, $P<0.001$.

4. Discussion

Here we present evidence to indicate that parotid and submandibular glands of NOD mice develop a progressive loss of nitric oxide synthase activity associated with a deficient secretion and signaling in response to VIP stimulation. This conclusion is based on the following observations: First, there was a significant decrease of nitric oxide synthase activity in salivary glands of NOD mice regardless of the sex and salivary gland studied that started past the 10th week of age and progressed thereafter to reach a complete loss of NOS activity in 16–19 weeks old mice. The constitutive nature of the isoforms of NOS involved was assessed in activity experiments run in the absence of calcium (EGTA experiments) and it was subsequently confirmed by immunoblotting assays where an altered expression of NOS I was observed. Second, VIP which has been proved to potentiate muscarinic-evoked saliva secretion as well as protein secretion by salivary glands in normal mice, failed to induce secretion in NOD mice either of aqueous or proteinaceous saliva. Third, along with the lower effect of VIP on secretion, there was a deficient response of both NOS activity and cAMP production to VIP in submandibular and parotid glands from NOD mice compared with control mice. Particularly in the case of cAMP levels, the poor effect of VIP seems to be due to an alteration at VIP receptor level rather than in the signaling pathways involving G protein coupling to adenylyl cyclase since isoproterenol through the same signal transduction

pathway acted similarly in either NOD and BALB/c salivary glands. Regarding the lack of effect of VIP on NOS activity, the most suitable explanation appears to be the apparent loss of enzyme activity observed in salivary glands of NOD mice at the optimal conditions tested. However, we cannot rule out that a decreased VIP receptor expression or a receptor desensitization process might also contribute to the effect.

The alterations of NOS I described here are similar to those found in submandibular glands of younger female NOD mice reported previously (Rosignoli et al., 2001). It is interesting to note that the loss of NOS activity observed in salivary glands from NOD mice progressively affected the glands in both males and females before mononuclear infiltration is evidenced suggesting that early changes in biochemical pathways within the gland not related to hormonal status may condition future structural and immunopathological damage of the glands. In this regard, the possibility that functional events affecting the target organ could drive an autoimmune response against the organ has been first proposed for a thyroiditis model in obese chicken (Roitt et al., 1998) and we have discussed this hypothesis in previous reports on a murine model of autoimmune myocarditis (Perez Leiros et al., 1994) and for younger NOD mice (Rosignoli et al., 2001). Similar conclusions have been also drawn from studies on apoptosis in salivary glands of NOD mice (Kong et al., 1998). The role of nitric oxide in neurotransmitter signaling has been extensively revised (Moncada et al., 1991; Dawson and Snyder, 1994) and the increasing importance of this diffusible mediator in several situations is highlighted by recent findings about the fine regulation of NOS expression through variations in the levels of nitric oxide itself that might favor the induction of one isoform of NOS over the others (Colasanti et al., 1997; Förstermann et al., 1998). Nitric oxide production is also controlled at the post-translational levels mainly through phosphorylation (Nathan and Xie, 1994). In reference to the overall secretory process, nitric oxide is known to promote salivary secretion in various in vivo and in vitro experimental designs (Lomniczi et al., 1998; Lohinai et al., 1999) and previous studies have demonstrated the expression of three isoforms of NOS in submandibular and parotid glands of normal mice (Rosignoli et al., 2001) and in labial glands from humans (Kontinnen et al., 1997). Thus, in the light of the above references, the identification of NOS I as the isoform affected in both parotid and submandibular glands of NOD mice reported here strongly confirms the role of nitric oxide from neural origin in saliva secretion and it also indicates that the relative abundance of NOS I over the other isoforms in salivary glands observed in immunohistochemical studies (Mitsui and Furuyama, 2000) might have a functional correlate.

Together with a defective NOS I, we presented evidence of a reduced response to VIP in major salivary glands of NOD mice. The effect was observed in nitric oxide production and cAMP levels of both glands and converged in a reduced protein and saliva secretion promoted by the neuropeptide. Although the lack of effect of VIP on NOS activity could be mostly explained by the defective activity of NOS in basal conditions, this is not the case for cAMP production. Certainly, the fact that isoproterenol was able to stimulate cAMP levels in salivary glands from either NOD and BALB/c mice argues against an altered Gs protein or adenylyl cyclase activity and rather supports the hypothesis that VIP receptor expression or receptor-Gs coupling might be responsible for the effect. Moreover, the possibility that adenylyl cyclase isoforms necessary for signal transduction have to be recruited from intracellular compartments on stimulation with VIP has been proposed by Hodges et al. (1997) and should be also taken into account in the present model. The concentration–response curves of cAMP in response to VIP are considered suitable to study receptor expression particularly when the receptor density is low even though the maximal response depends on various pharmacological parameters of the receptor and on the cell type studied (Robberecht and Waelbroeck, 1998). Thus, the comparison of the curves for cAMP accumulation in NOD and control mice shown here are strongly supportive of a reduced expression and/or desensitization of VIP receptors in this model. Previous reports on decreased expression of both β-adrenergic and muscarinic acetylcholine receptors in salivary glands from NOD mice have considered that the lower expression of receptors was responsible for the lower response of the glands to the agonist (Hu et al., 1994; Yamamoto et al., 1996). The authors have shown that diabetic NOD mice presented a decreased population of both autonomic receptors resulting in alterations of their signaling cascades while in prediabetic 6-month-old mice the differences were less pronounced and mostly affected submandibular glands. It is worth pointing out that, in contrast to our results, prediabetic mice in those studies already presented a diminished secretory response to pilocarpine alone indicating that probably they were at a more advanced stage of disease than 16–19 weeks old NOD mice used here. Regarding neuropeptide receptors associated to autonomic secretion in NOD mice, a lower secretory response to VIP has been previously shown in prediabetic and diabetic NOD mice and the effect could not be explained by changes in neuropeptide levels within the glands (Yamamoto et al., 1997). Hence, to our knowledge, this is the first report on defective signaling of VIP receptors in salivary glands of NOD mice confirming and extending previous observations from other researchers and from our own work.

Among the various events that could affect the secretory response to VIP in salivary glands of NOD mice, the action of mediators of the autoimmune response should be taken into account. Certainly, several reactions mediated by autoantibodies or cytokines can contribute to a defective function of target tissues during an autoimmune response. In fact, we have reported on desensitization

and internalization of M_2 muscarinic acetylcholine receptors induced by autoantibodies from chagasic patients (Pérez Leirós et al., 1997) as well as the ability of autoantibodies from SS patients to interfere M_1 and M_3 muscarinic receptor signaling in salivary and lacrimal glands (Perez Leiros et al., 1999; Bacman et al., 1998). Moreover, evidence to indicate that the second extracellular loop of these receptors was recognized by autoantibodies has been provided (Goin et al., 1997; Perez Leiros et al., 1999). Regarding cytokines and autonomic nervous system dysfunction, interferon γ and IL-2 were shown to alter heart muscarinic and β adrenergic activation, respectively, by modifying receptor affinity for the agonists and their signaling pathways (Borda et al., 1991; Sterin-Borda et al., 1996). In support of this observation, a role for interferon γ has been proposed in the progression to cardiac muscarinic dysfunction in a model of autoimmune myocarditis (Perez Leiros et al., 1998) as well as the involvement of various cytokines in NOD sialadenitis (Yamano et al., 1999). Finally, VIP was shown as a modulator of the immune response through its selective action on different VIP receptor subtypes expressed on lymphocytes and macrophages (Goetzl et al., 1998). Also, an anti-inflammatory action in vitro and in vivo has been proposed (Pozo et al., 2000). In the light of these reports and the observations described here, it is tempting to speculate that VIP levels within the glands could have a role in regulating not only VIP receptor expression and activity in acinar and nervous cells but also the activity of receptors on resident immune cells as well as the recruitment and activation of leukocytes. Interestingly, VIP levels appeared decreased in submandibular glands of prediabetic NOD mice but increased in parotid glands with respect to controls (Yamamoto et al., 1997), suggesting that neuroimmune mechanisms involving VIP receptors on other non-exocrine cells might modulate the overall process of secretion.

Assays for the characterization and the identification of VIP receptor subtypes in exocrine glands have been conducted mainly in rat glands. Hence, by means of confocal microscopy using antibodies against VIP_1 ($VPAC_1$) and VIP_2 ($VPAC_2$) receptor subtypes, both receptors were found on the basolateral membranes of acinar and ductal cells and on myoepithelial cells of lacrimal glands (Hodges et al., 1997), whereas the coexistence of VIP receptor subtypes was suggested from experiments in which a VIP antagonist blocked the VIP enhancement of saliva volume but not the increase in protein in submandibular glands (Turner and Camden, 1992) and from competition binding assays with (^{125}I-VIP) and different VIP related peptides in submandibular and parotid glands (Turner and Bylund, 1987; Inoue et al., 1985; Dehaye et al., 1985). Further experiments are needed to identify the VIP receptor subtypes involved in the effects shown here as well as the immune or exocrine nature of the VIP receptor bearing cells participating in those effects.

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