Lack of nitric oxide-mediated regulation of amylase secretion stimulated by VIP in parotid glands of NOD mice

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Abstract

The non-obese diabetic (NOD) mouse is chosen among other experimental models to study autoimmune sialadenitis resembling Sjögren’s syndrome (SS), because of its unique characteristic of developing salivary dysfunction. Based on the deep loss of nitric oxide synthase (NOS) activity in parotid glands of NOD mice observed from early stages of disease and the inhibitory effect of nitric oxide (NO) donors on amylase secretion in normal salivary glands, our goal was to investigate whether parotid glands from NOD mice lacking NOS activity presented this regulatory mechanism of amylase secretion. We found that parotid glands from NOD mice lack nitric oxide-mediated regulation of amylase secretion in response to VIP stimulation. The lack of regulation might be assigned to the loss of NOS activity as derived from the results with NOS inhibitors and increasing concentrations of VIP. These functional differences observed in NOD vs. BALB/c parotid glands occur in the absence of immune infiltrates in exocrine tissue, and it is not related to cAMP accumulation. NO-mediated regulation of amylase secretion was not observed in BALB/c submandibular glands to the same extent as described in parotid glands and was absent in submandibular glands of NOD mice.

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1. Introduction

Sjögren’s syndrome (SS) is a chronic autoimmune disorder of unknown etiology characterized by a severe dryness of the mouth and the eyes [1–3]. The mild infiltration of salivary glands cannot fully account for the severe loss of secretory function supporting the hypothesis that neural rather than immune mechanisms have a role in the pathogenesis of this disease [3]. The non-obese diabetic (NOD) mouse is chosen among other models to study autoimmune sialadenitis resembling Sjögren’s syndrome (SS)—especially SS associated to other connective tissue diseases (secondary SS)—because of its unique characteristic of developing a deep loss of
secretory function [4]. As reported in patients, there is a poor correlation between the moderate sialadenitis and the reduction in saliva secretion. This is especially observed in parotid glands where a lower saliva flow rate was observed in the complete absence of lymphomononuclear infiltrates [5]. In addition, we have described a loss of nitric oxide (NO) production and signaling in salivary glands of NOD mice developing SS-like symptoms that precedes the autoimmune response [6,7]. Taken together, these observations prompted us to suggest that early modifications in organ or cellular homeostasis might predispose the salivary glands to the autoimmune response. Amylase secretion promoted by parasympathetic stimulation involves vasoactive intestinal peptide (VIP) receptors [8] which are coupled to different signaling pathways, including the L-arginine–nitric oxide signaling pathway [7]. NO is involved in carbachol-stimulated amylase secretion in vitro [9], and a downregulatory role of nitric oxide donors on amylase secretion was observed in rats in vivo [10]. Based on these observations, we hypothesized that parotid glands from NOD mice with a lower NOS activity might present a defective NO-mediated regulation of amylase secretion. We also analyzed whether such functional changes were associated with histological damage and mononuclear infiltrates in salivary glands. We present evidence to indicate that NO production coupled to high concentrations of VIP inhibits amylase secretion in parotid glands of normal BALB/c mice but not in NOD mice. Submandibular glands from BALB/c mice showed a modest NO-mediated regulatory effect compared with parotid glands that was absent in NOD mice. This defect in regulation occurs in the absence of infiltrates in both glands of NOD mice, and it does not involve cAMP accumulation.

2. Materials and methods

2.1. Animals

NOD and BALB/c female mice were bred and maintained in the Central Animal Care facility at the School of Exact and Natural Sciences, University of Buenos Aires. NOD and BALB/c mice of 15 weeks old were used due to the fact that at this age, there are no signs of histological damage of the glands, but NOS activity is already decreased in submandibular and parotid glands [7]. They were tested for blood glucose levels using the glucose oxidase method in 20-μl samples of NOD and control sera (Wiener Lab., Rosario, Argentina). Blood was drawn by retro-orbital puncture every week starting at 12 weeks of age and before being used. NOD mice used throughout were considered pre-diabetic as their values of serum glucose (1.0±0.1 g/l, n=14) did not significantly differ from those of control mice (0.9±0.1 g/l, n=14). Mice were fasted overnight with water ad libitum before being used, and all studies were conducted according to standard protocols of the Animal Care and Use Committee of the School of Exact and Natural Sciences, University of Buenos Aires.

2.2. Nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in parotid glands from fasted mice using L-[U-14C]-arginine as substrate as described earlier [7,11]. Whole glands were incubated with 0.2 μCi L-[U-14C]-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% CO₂ in O₂ at 37 °C for 30 min. Then, tissues were homogenized in 20 mM HEPES pH 5.5 with 0.5 mM EDTA, 1 mM dithiothreitol (DTT) and 0.5 mM EGTA. [14C]-Citrulline was separated by ionic exchange chromatography on a Dowex AG 50W-X8 resin (Bio-Rad) and radioactivity counted in a β-spectrometer. NOS activity was calculated as total activity minus that measured in the presence of 500 μM L-N³G-monomethyl arginine (LNMMA) (Sigma, MO, USA) and expressed as fmol [14C]-citrulline/mg tissue.

2.3. Amylase secretion assay

Amylase secretion was determined as previously reported [9]. Parotid or submandibular glands from fasted mice were incubated in KRB without glucose for 30 min at 37 °C in the presence of the indicated final concentrations of VIP for the last 15 min and when used, LNMMA (500 μM) or 100 μM trifluoperazine (TFP) (Sigma) were included from the beginning of the incubation time. Once incubation was
finished, the medium was transferred to other tubes for amylase activity determination, and the tissues were homogenized in 50 mM phosphate buffer at 4 °C, spun down at 10,000×g for 20 min, and the supernatants were used for amylase activity assay. One unit of amylase was defined as the activity of amylase that released 1 mg of maltose per minute at 20 °C [12]. Results were expressed as % release of amylase calculated as the ratio between amylase activity in the incubation medium and total amylase (medium plus homogenate).

2.4. cAMP determination

Adenosine 3’,5’-cyclic monophosphate (cAMP) accumulation was determined in parotid and submandibular glands from fasted mice by means of a radioimmunoassay with anti-3’,5’-cAMP antisera kindly provided by Dr. A.F. Parlow from the National Hormone and Pituitary Program (USA) and [125I]-cAMP (>2200 Ci/mmol) labelled by Dr. Omar Pignataro from the IBYME (Buenos Aires, Argentina). Samples were prepared by incubating whole glands for 30 min in 1 ml KRb with 100 μM 3-isobutyl-1-methylxanthine gassed with 5% CO2 in O2. VIP was added in the last 15 min at the final concentrations indicated and, when used, 500 μM LNMMA was included from the beginning of the incubation time. Glands were homogenized in ethanol and after evaporation, residues were dissolved in 50 mM sodium-acetate buffer pH 6.2 for subsequent cAMP determination, and results were expressed in fmol/mg tissue wet weight.

2.5. Histological studies

Parotid or submandibular glands from NOD mice were embedded in paraffin wax and sections of 4 μm were cut, placed on siliconized glass slides and stained with haematoxylin–eosin as reported previously [7]. Slices were observed, and the number of ducts quantified in a survey of 20 fields for each slice and the results were expressed as ducts/field.

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.

3. Results

3.1. Nitric oxide synthase activity and amylase secretion in salivary glands stimulated by VIP

Fig. 1 shows the stimulatory effect of VIP on nitric oxide synthase activity in parotid glands of BALB/c mice with a maximal effect at 10^-8 M of the neuropeptide. In contrast, NOD parotid glands show a lack of both basal and VIP-stimulated NOS activity. A similar concentration–response curve was obtained for submandibular glands of BALB/c mice as we have reported previously [6,7], where 10^-8 M VIP stimulated NOS activity, though to a lower extent than in parotid glands shown here. In addition, the lack of basal and VIP-stimulated NOS activity in submandibular glands of NOD mice has been already reported [6,7]. In order to analyze the simultaneous effect of VIP on amylase secretion and the role of NO in this effect, we determined amylase release in parotid glands at the same concentration range of VIP in the presence and absence of the NOS inhibitor LNMMA. As it can be
seen in Fig. 2a, VIP inhibited amylase secretion at the same concentration that stimulated NOS activity in control mice, but this effect was not seen in NOD mice. The inhibitory effect on amylase is evidenced not only by the decrease in amylase secretion but also by the reversal with the NOS inhibitor LNMMA and the calmodulin inhibitor TFP (Fig. 2b). Consistent with the lack of NOS activity observed, parotid glands of NOD mice showed a higher basal release of amylase, and no down-regulation was observed at any VIP concentration tested (Fig. 2a). In addition, Fig. 2b shows that LNMMA and TFP had no effect on NOD amylase secretion pointing to the lack of a NO-mediated modulatory pathway of proteinaceous secretion in parotid glands from NOD mice. Submandibular glands were also assayed for amylase release, and as can be seen in Table 1, 10^{-8} M VIP did not inhibit amylase secretion as observed in parotid glands, while the inhibition of NOS activity with LNMMA only induced a modest increase of amylase release at this concentration of VIP. Again, as observed in NOD parotid glands, there was no

Fig. 2. Amylase secretion in response to VIP and the role of nitric oxide in the effect. (a) Parotid glands of BALB/c and NOD mice were incubated with different concentrations of VIP and amylase secretion was calculated as described. Values are the mean±S.E.M. of at least five glands. *P<0.05 vs. basal or corresponding VIP concentration in BALB/c mice. **P<0.05 vs. basal in BALB/c mice. (b) Parotid glands were incubated with or without (basal) 10^{-8} M VIP in the presence or absence of 500 μM LNMMA or 100 μM TFP, and amylase secretion was calculated as above. Values are means±S.E.M. of at least four glands. *P<0.05 vs. 10^{-8} M VIP.

<table>
<thead>
<tr>
<th>Submandibular glands</th>
<th>Amylase (% of release)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>NOD</td>
</tr>
<tr>
<td>Basal</td>
<td>342±69</td>
</tr>
<tr>
<td>LNMMA</td>
<td>511±83</td>
</tr>
<tr>
<td>TFP</td>
<td>194±51</td>
</tr>
<tr>
<td>VIP 10^{-8} M</td>
<td>644±82</td>
</tr>
<tr>
<td>VIP 10^{-8} M+ LNMMA</td>
<td>966±74*</td>
</tr>
<tr>
<td>VIP 10^{-8} M+ TFP</td>
<td>520±129</td>
</tr>
</tbody>
</table>

Submandibular glands were incubated in KRB in the presence or absence of 500 μM LNMMA or 100 μM TFP as described in Materials and methods with 10^{-8} M VIP as indicated in the table. When used, inhibitors were included from the beginning of the incubation time. Values are the means±S.E.M. of at least four experiments with glands from different mice.

* P<0.05 vs. VIP 10^{-8} M.
Table 2
Effect of VIP on cAMP accumulation in NOD and control salivary glands

<table>
<thead>
<tr>
<th></th>
<th>BALB/c</th>
<th>NOD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parotid glands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>179±19</td>
<td>223±26</td>
</tr>
<tr>
<td>VIP 10^{-6} M</td>
<td>275±87</td>
<td>300±87</td>
</tr>
<tr>
<td>VIP 10^{-7} M</td>
<td>528±83*</td>
<td>337±23*</td>
</tr>
<tr>
<td>VIP 10^{-7} M+ LNMMA</td>
<td>373±69</td>
<td>264±91</td>
</tr>
<tr>
<td><strong>Submandibular glands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>233±14</td>
<td>227±12</td>
</tr>
<tr>
<td>VIP 10^{-6} M</td>
<td>473±93</td>
<td>225±37</td>
</tr>
<tr>
<td>VIP 10^{-7} M</td>
<td>499±22*</td>
<td>177±72</td>
</tr>
<tr>
<td>VIP 10^{-7} M+ LNMMA</td>
<td>702±121</td>
<td>294±39</td>
</tr>
</tbody>
</table>

Parotid or submandibular glands were incubated in KRB in the presence of 100 μM IBMX as described in Materials and methods with the concentrations of VIP indicated in the table. When used, 500 μM LNMMA was included from the beginning and cAMP accumulation was determined by RIA. Values are the means±S.E.M. of at least four experiments with different glands.

* P<0.05 vs. basal of the same mice.

3.2. cAMP accumulation in response to VIP

In an attempt to further study the mechanisms underlying NO-mediated amylase reduction in normal mice glands, we investigated the accumulation of cAMP in response to VIP and the effect of the NOS inhibitor LNMMA. As shown in Table 2, VIP stimulated cAMP accumulation in parotid glands from BALB/c mice and to a lower extent in glands from NOD mice. Similar concentration–response curves of VIP were obtained in submandibular glands of BALB/c mice, but no effect of VIP was seen in NOD submandibular glands. The inhibition of NOS with LNMMA did not modify cyclic nucleotide accumulation in either gland suggesting that this effect is not dependent on nitric oxide production in BALB/c or NOD salivary glands stimulated with VIP.

3.3. Histological studies of NOD and BALB/c parotid glands

Fig. 3 shows haematoxylin–eosin-stained slices of parotid glands from NOD and control mice. A similar epithelial cell structure and distribution of acini and ducts was observed in parotid glands of both mice and no immune cell infiltrates could be found. In addition, the relationship acini–ducts did not vary in parotid glands. Histological studies of submandibular glands showed no immune infiltrates or morphological alterations of epithelial cells in acini and ducts in NOD vs. BALB/c mice (data not shown).

4. Discussion

We present evidence to indicate that parotid glands from NOD mice lack nitric oxide synthase activity and NO-mediated regulation of amylase secretion in response to VIP stimulation. This functional alteration in NOD parotid glands at 15 weeks of age occurs in the absence of immune...
infiltrates in the exocrine tissue, and it is not related to cAMP accumulation. These conclusions are supported by the following observations. First, VIP stimulated NOS activity in control BALB/c parotid glands, but there was no basal or VIP-stimulated activity of NOS detectable in NOD mice. Second, BALB/c parotid glands showed a lower secretion of amylase in response to VIP that was mediated through NO since the inhibition of NOS activity with TFP or LNMMA reversed the effect. Consistent with this, the effective concentration of VIP able to inhibit amylase in BALB/c mice (10⁻⁸ M) was the same that maximally stimulated NOS. In contrast, parotid glands of NOD mice showed a higher basal release of amylase, and at the same concentrations of VIP, no regulation was observed. A lower effect of endogenous NO-mediated inhibition of amylase was observed at 10⁻⁸ M VIP in submandibular glands of BALB/c mice but not in NOD mice. The lack of regulation in both salivary glands of NOD mice might be assigned to the loss of NOS activity as derived from the results with NOS inhibitors, and it seemed not to be related to cAMP accumulation. Third, these functional differences observed in NOD vs. BALB/c salivary glands occur in the absence of lymphomonomuclear infiltrates in either gland. Though it is accepted that NO is involved in salivary flow rate stimulation [13,14], its role in proteaceous secretion has not been fully understood. Our results in normal BALB/c parotid glands used as a control of NOD mice glands are in line with previous reports of an inhibitory effect of NO donors on amylase secretion and an increase of amylase secretion with a NOS inhibitor in rats [10]. The fact that in normal parotid glands, VIP stimulated cAMP at higher concentrations than those necessary to stimulate NOS and that the effect was not modified by the NOS inhibitor LNMMA strongly suggests that cyclic nucleotide accumulation is independent of NO. The same was observed in submandibular glands where LNMMA had no effect on cAMP accumulation either. Confirming this, the concentration of VIP that stimulated NOS and reduced amylase secretion (10⁻⁸ M) were ineffective in cAMP accumulation, and those that stimulated cAMP (10⁻⁷ M) had no effect on amylase. On the other hand, the independence of cAMP from NO-mediated pathways is further supported by the increase of cAMP levels at 10⁻⁷ M VIP, a concentration that was ineffective in NOS activity and the effect of 10⁻⁸ M VIP in cAMP accumulation in a situation where no activity of NOS was detected as is the case of NOD mice. It is well known that the main signaling pathway underlying amylase secretion through VIP receptors in normal parotid cells proceeds via cAMP [15,16]. Concentrations of VIP higher than those used here were shown to stimulate amylase secretion in rat parotid glands in vitro [17], and most reports on VIP stimulation of cAMP at concentrations similar to those used here refer to dispersed cultured parotid acinar cells rather than whole glands [15,16]. Comparing in vivo concentrations of VIP in the extracellular fluid of gastrointestinal tract after parasympathetic stimulation (about 10⁻¹⁰ M) [18] with the concentration range for ex vivo VIP-induced NO-mediated regulation of amylase shown here, it is conceivable that such an inhibitory mechanism on amylase secretion might protect the exocrine cells from secreting high amounts of protein at concentrations of VIP higher than physiological. Interestingly, immunopathological conditions are associated with a higher release of VIP in various tissues [19]. NOS expression in labial minor salivary glands of Sjögren’s patients has been studied previously [20]. By means of immunocytochemical analysis of these glands, the authors reported on a very sparse distribution of NOS I-containing nerve fibers and parallel changes in NOS III expression, but the endothelial isoform show a strong immunoreactivity in focal inflammatory infiltrates of SS labial salivary glands. Regarding NOS II, it was detected in SS salivary glands but also to some extent in healthy controls [20]. These results are in accordance with those described here and previously [6,7] for NOD mice and point to the potential role of impaired neurotransmitter receptor signaling in the ulterior salivary dysfunction. As suggested in other organ-specific autoimmune models [21,22], early functional alterations in NOD mice exocrine glands might predispose them to the autoimmune response. In line with this hypothesis, we first described a lower salivary flow with loss of basal NOS activity in submandibular glands of NOD mice that was not associated with immune infiltrates [6] and evidence of altered expression of metalloproteases [23] and autonomic receptors [24] has
been provided. The lack of NO-mediated regulation of amylase secretion reported here clearly indicates that the loss of NOS activity has a functional correlate that might affect glandular homeostasis. The effect is consistent with the loss of NOS activity; it is previous to morphological alterations of acini and ducts and occurs in the complete absence of lymphomononuclear infiltrates in both glands. Finally, the fact that basal and VIP-stimulated amylase secretion were higher in NOD vs. normal mice, that TFP and LNMMA had no effect on amylase secretion in NOD mice, and that VIP only slightly stimulated cAMP accumulation suggests that other second messengers might have taken the control of VIP-stimulated amylase secretion in NOD mice. Further studies are necessary to investigate if a different intracellular milieu might condition VIP receptor signaling and regulation in NOD glands.

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References


