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Alterations in Nitric Oxide Synthase Activity and Expression in Submandibular Glands of NOD Mice

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The non-obese diabetic (NOD) mouse model of autoimmune sialadenitis offers the possibility of studying the L-arginine/nitric oxide signaling pathway in salivary glands in basal and neurotransmitter-stimulated conditions and, thus, of analyzing the neural control of the secretory process in the target organ. The purpose of this study was to explore putative alterations in the activity and expression of nitric oxide synthase (NOS) in submandibular glands of NOD mice in relation to parotid glands and unrelated tissues. Here we report that NOD mice with incipient signs of secretory dysfunction presented a marked decrease in basal and vasoactive intestinal peptide (VIP)-stimulated NOS activity and a differential expression of NOS I in submandibular glands compared to control BALB/c mice. Similar alterations in NOS I were found in parotid glands but not in brain or spleen of NOD mice. No differences between NOD and controls appeared in NOS II and NOS III expression in any of the tissues studied. © 2001 Academic Press

Key Words: **nitric oxide synthase; NOD; VIP; salivary glands; autoimmune sialadenitis; NOS isoforms.**

INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune disorder characterized by histological and functional alterations of exocrine glands that result in a severe dryness of mouth and eyes (1–3). Though it has been proposed that secretory dysfunction is due to structural damage of acinar cells in salivary glands, the focal and sparse distribution of lymphomononuclear infiltrates in glands of patients correlates poorly with the marked impairment of saliva flow (3). This observation has prompted the authors to suggest that glandular infiltration cannot fully account for SS symptoms and that alterations in neural regulatory pathways may have a role in the pathogenesis of *sicca syndrome*. In support of this hypothesis, evidence of a diminished innervation of salivary glands has been reported in patients with Sjögren's syndrome (4, 5), and we have previously shown that autoantibodies from Sjögren's syndrome patients interacted with muscarinic acetylcholine receptor signaling pathways in exocrine glands, modifying normal physiologic responses (6–8).

The *non-obese diabetic* (NOD) mouse represents one of the best models for studying autoimmune sialadenitis resembling some features of Sjögren's syndrome, especially SS associated with other connective tissue diseases (secondary SS) (9–11). Of note is that a poor correlation between lymphomononuclear infiltrates and a reduction in saliva secretion was found in parotid glands of these mice (12), indicating that functional rather than structural changes lead to secretory dysfunction in NOD mice, similarly to SS patients.

Among the various signaling pathways involved in the nervous control of saliva flow, nitric oxide might be assigned a critical role based on its dual behavior as a physiological messenger of several neurotransmitter receptors (13) and as an inflammatory mediator in a growing variety of diseases (14). In this regard, it has been documented that the nitric oxide signaling pathway is involved in salivary secretion (15–17), but its participation in autoimmune sialadenitis still remains unclear. Nitric oxide is synthesized from L-arginine by a family of nitric oxide synthase (NOS) enzymes that occur in three different isoforms in mammalian tissues, each subject to fine transcriptional, posttranscriptional, and/or posttranslational regulation (18, 19). The NOD mouse model offers the possibility of studying the L-arginine/nitric oxide signaling pathway in basal and neurotransmitter-stimulated conditions, thus allowing to analysis of neural control of the secretory process in the target organ. Therefore, the purpose of this study was to explore putative changes in the activity and expression of nitric oxide synthase in submandibular glands of NOD mice at an early stage of disease. Here we report that NOD mice with incipient signs of secretory dysfunction presented a marked decrease in basal and stimulated nitric oxide synthase activity and a differential expression of the neural iso-

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form of the enzyme (NOS I) in submandibular glands with respect to control BALB/c mice. Similar alterations were found in parotid glands but not in brain or spleen of these mice. On the other hand, there were no changes in the expression of NOS II and NOS III between NOD and control mice, neither in salivary glands nor in the other tissues studied.

MATERIALS AND METHODS

Animals

Female NOD and BALB/c mice were bred and maintained under specific pathogen-free conditions in the Central Animal Care facility at the University of Buenos Aires (Ciudad Universitaria, Buenos Aires). NOD mice (12–14 weeks old) 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 prediabetic, as their values of glucose (140 $\pm$ 9 mg/dl, n = 5) did not significantly differ from those of control mice (129 $\pm$ 9, n = 5). Salivary glands from NOD mice used throughout showed no signs of histological damage or mononuclear cell infiltration, as found on slices from glands embedded in paraffin and stained with haematoxylin–eosin and reported previously (20). Also, NOD mice did not present circulating antibodies against salivary gland proteins assessed by immunoblotting as previously described (7). 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.

Salivary Flow Rates and Amylase Secretion

Total saliva was collected after stimulation of secretion with either the muscarinic agonist pilocarpine (Sigma Chemical Co., St. Louis, MO) (50 μg pilocarpine/100 g weight) injected intraperitoneally in mice fasted overnight or pilocarpine plus VIP (Sigma) (10 μg vasoactive intestinal peptide (VIP)/100 g weight). By means of a micropipette, saliva accumulated in the oral cavity was driven through 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 body wt. The concentrations of protein and amylase in saliva were determined in an aliquot of the fluid collected from each mouse by the methods of Lowry (21) and Bernfeld (22), respectively. The results were expressed in micrograms protein/μl of saliva collected per minute per 100 g body wt or amylase units/ml of saliva/min/100 g body wt. One unit of amylase was defined as the activity of amylase that released 1 mg of maltose per minute at 20°C (22).

Nitric Oxide Synthase Activity

NOS activity was measured in submandibular and parotid glands from fasted mice using L-[U-^{14}C]arginine as substrate as described earlier (8, 23). 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 for 30 min at 37°C. Specific NOS activity was calculated as total activity minus that measured in the presence of 50 μM L-N^G-monomethylarginine (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 stimuli (VIP or carbachol, Sigma) were added at the final concentrations indicated for the last 15 min.

Immunoblotting

Submandibular and parotid glands, cerebral cortex, and spleen from fasted mice were excised 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 (7, 23). Once centrifuged at 5000g for 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), and molecular weight standards (Amersham Pharmacia Biotech) were subjected to 7.5% SDS–PAGE, transferred to nitrocellulose membranes, and revealed with specific monoclonal antibodies against NOS I, II, and III (Transduction Labs) using an alkaline-phosphatase conjugate (Sigma), as we have described earlier (7).

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 $P < 0.05$.

RESULTS

Basal and Stimulated Nitric Oxide Synthase Activity in Submandibular Glands of NOD and Control Mice

Figure 1 shows the activity of total nitric oxide synthase in submandibular glands from NOD and control

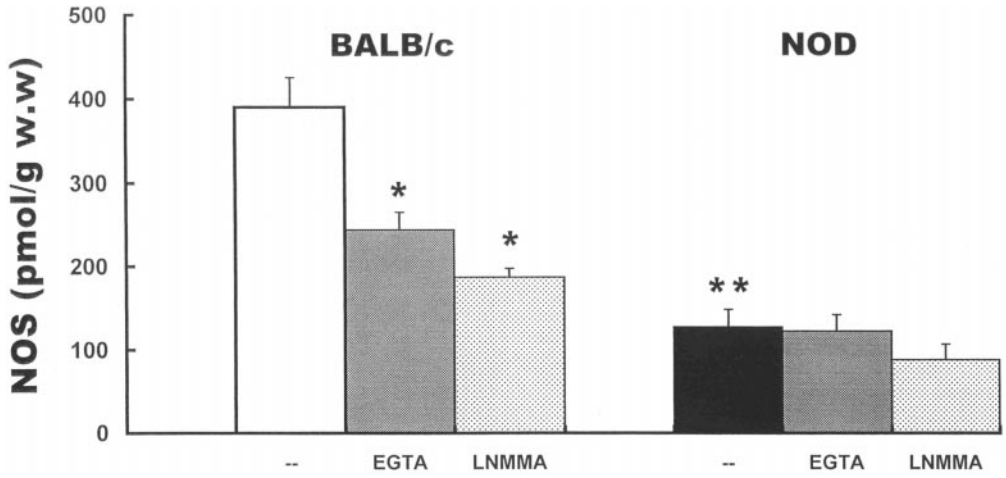

FIG. 1. NOS activity in submandibular glands of NOD and control mice. Submandibular glands of NOD and BALB/c mice were incubated with L-[U- ^{14}C]arginine and the activity of NOS was determined as described under Materials and Methods. Assays were carried out in the absence (—) and presence of 50 μM L-N^G-monomethylarginine (LNMMA) or 5 mM EGTA (EGTA) as indicated in the graph. Values represent the means ± SEM of at least five experiments. ***** Significantly different from basal BALB/c without additions, $P < 0.05$; ****** significantly different from basal BALB/c without additions, $P < 0.01$.

BALB/c mice. A significant decrease in total NOS activity could be seen in NOD compared with BALB/c mice. Total NOS activity includes the activity of the three isoforms; thus, to further explore whether this marked decrease in NOS activity was due to constitutive (calcium-dependent) or inducible (calcium-independent) isoforms, we carried out the assays in the absence of calcium. As can be seen in Fig. 1, constitutive isoforms are predominant in submandibular glands of BALB/c mice, as EGTA completely inhibited specific NOS activity. In contrast, NOS activity in NOD mice showed no difference whether in the presence or absence of calcium, indicating either altered expression of constitutive isoforms or a strong posttranslational down-regulation of constitutive NOS, as that mediated by protein kinase C (PKC) in submandibular glands, which we have previously reported (23). Parotid glands of NOD mice showed diminished NOS activity in basal conditions similar to submandibular glands (Table 1). Again, the NOS isoforms active in parotid glands of BALB/c mice and affected in NOD mice seemed to be constitutive isoforms, as derived from EGTA assays (Table 1).

In order to see whether the low basal activity of NOS observed in salivary glands of NOD mice was sensitive to neurotransmitter receptor-coupled activation, we incubated the glands with VIP and carbachol, both stimuli responsible for saliva secretion, and determined NOS activity. As shown in Figs. 2A and 2B, NOS in submandibular glands from NOD mice was unresponsive to increasing concentrations of VIP or carbachol at the concentrations tested, in contrast to that in normal BALB/c glands. Similarly, parotid glands from NOD mice also failed to respond to stimuli as did the glands from control mice (Table 1).

Expression of NOS Isoforms in Submandibular Glands of NOD and Control Mice

To investigate whether the lack of specific basal NOS activity in NOD submandibular glands was associated with an altered expression of the enzyme and to identify which particular isoform was involved, we carried out immunoblotting assays with antibodies specific for NOS I, II, and III on extracts of submandibular glands from both NOD and control BALB/c mice. Figure 3

TABLE 1

Basal and Stimulated Activity of NOS in Parotid Glands of NOD Mice

	NOS activity (pmol/g)	
	BALB/c	NOD
Basal	852 ± 78	431 ± 50*
L-NMMA	395 ± 72*	294 ± 33
EGTA	613 ± 34*	310 ± 81
VIP (1 nM)	1351 ± 53*	446 ± 42
Carbachol (10 μM)	452 ± 51*	511 ± 36

Note. Parotid glands of 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 50 μM L-N^G -mono-methyl arginine (L-NMMA), 5 mM EGTA, VIP or carbachol as indicated. Values represent the mean ± SEM of at least 3 experiments.

* Significantly different from basal BALB/c, $P < 0.05$.

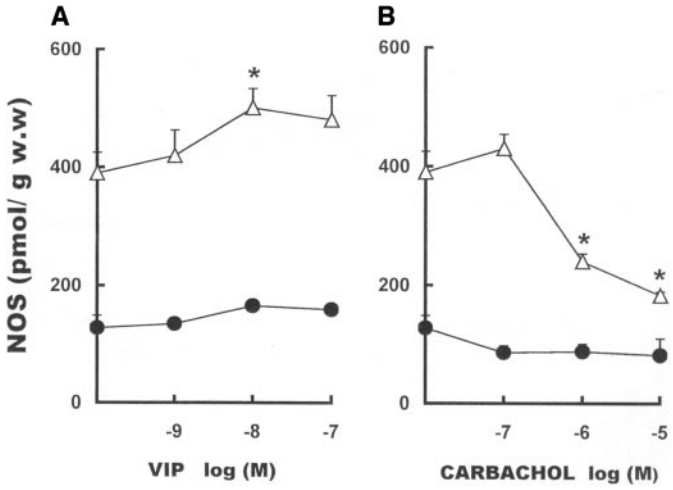

FIG. 2. Effect of VIP and carbachol on NOS activity in submandibular glands of NOD and control mice. Submandibular glands of NOD (●) and BALB/c (△) mice were incubated with the substrate and increasing concentrations of either VIP (A) or carbachol (B) for subsequent determination of NOS, as indicated under Materials and Methods. Values are the means ± SEM of at least five experiments. * Significantly different from the basal value (0, the absence of VIP or carbachol) of the corresponding curve, $P < 0.05$.

shows the bands corresponding to NOS I, II, and III in submandibular glands of BALB/c and NOD mice. The expression pattern of NOS I in control BALB/c submandibular glands showing a prominent band at a Mr lower than 150 kDa has been already described (24). Expression of the neural isoform in submandibular glands of NOD mice differed from that in controls, with no changes in the other two isoforms. In order to examine whether this effect was exclusive to submandibular glands or could be found in other salivary glands, in nervous tissue, or in less-innervated tissues, we prepared extracts of parotid glands, cerebral cortex, and spleen from NOD and control mice. As shown in Fig. 3, while a similar alteration of NOS I appeared in parotid glands, there were no modifications in this isoform in brain or spleen. To assess whether this alteration of NOS I in salivary glands was paralleled by an altered expression of the other two isoforms of the enzyme, we investigated the expression of NOS II and III in the same tissues; Fig. 3 shows that there were no changes in NOD and BALB/c mice in NOS II and III expression in both salivary glands and unrelated tissues.

Saliva Flow Rates and Amylase Secretion in NOD and BALB/c Mice

In order to investigate the current secretory function of prediabetic NOD mice, we determined saliva flow rate and protein and amylase secretion under basal and stimulated conditions, as described under Materials and Methods. Figure 4 shows that NOD mice presented a lower saliva flow rate than BALB/c control mice when stimulated with both pilocarpine and VIP, while no significant differences could be detected in the rate between NOD and control mice stimulated with pilocarpine alone. On the other hand, the concentrations of both protein and amylase in saliva were not reduced in NOD mice by either pilocarpine or the mix of pilocarpine and VIP at this early stage of disease.

DISCUSSION

Here we present evidence indicating that both the activity and the expression of nitric oxide synthase are altered in submandibular glands of NOD mice compared to in those of control BALB/c mice. The activity of total NOS was strongly diminished in NOD submandibular glands at basal conditions, with calcium-dependent isoforms contributing prominently to the effect, as revealed when this cation was absent. The lack of activity of constitutive NOS in NOD submandibular glands was also evidenced following muscarinic acetylcholine or VIP receptor activation, known as the most relevant nervous stimuli for saliva secretion, and pro-

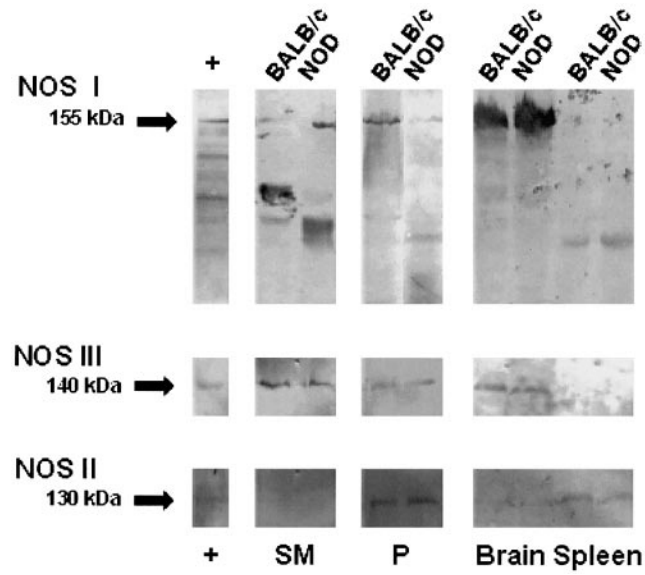

FIG. 3. Expression of NOS isoforms in submandibular glands and other tissues of NOD and BALB/c mice. Tissue extracts from control BALB/c mice and from NOD mice were obtained as described under Materials and Methods. Extracts from submandibular glands (SM), parotid glands (P), cerebral cortex (Brain), and spleen (Spleen) (100 μg protein/lane) and positive controls for each isoform (+) were subjected to 7.5% SDS–PAGE and then to immunoblotting assays with specific monoclonal antibodies against NOS I, III, and II isoforms. The arrows indicate the bands corresponding to each isoform, with the relative molecular weights in kDa. Results shown are representative of at least five similar experiments.

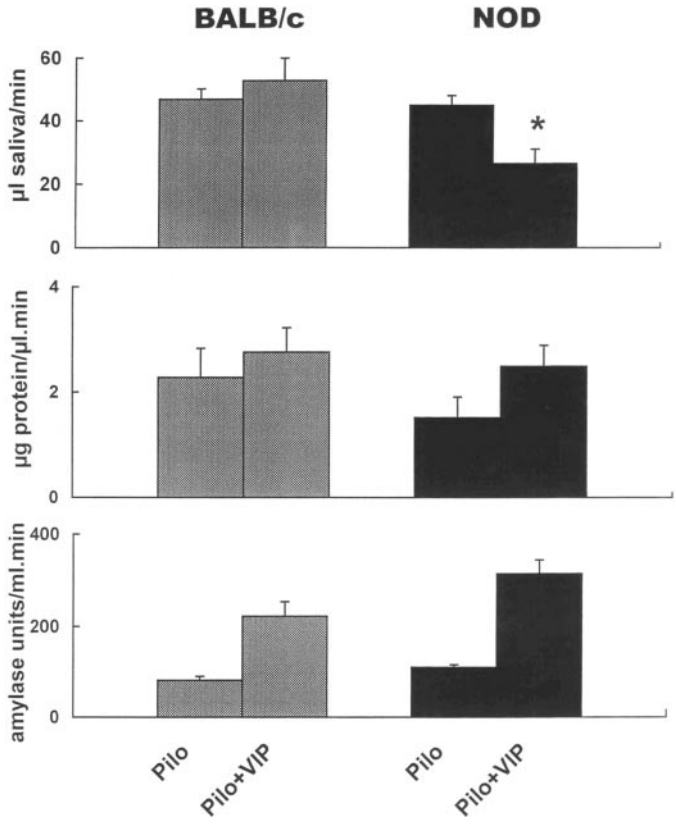

FIG. 4. Salivary flow rate, protein secretion, and amylase secretion of NOD and control mice. NOD and BALB/c mice fasted overnight were injected with pilocarpine (Pilo) or a mixture of pilocarpine and VIP (Pilo + VIP), and saliva was collected for 12 min following injection, as indicated under Material and Methods. Results shown are the means ± SEM of at least five experiments. * Significantly different from Pilo + VIP in BALB/c mice, $P < 0.05$.

vided the fact that both signal through nitric oxide-mediated pathways involving constitutive isoforms of NOS (23, 25). Certainly, both carbachol and VIP failed to modify NOS activity in NOD mice as they did in the BALB/c controls, confirming the constitutive nature of the isoforms of NOS affected in these glands. Interestingly, the effect was also observed in another salivary gland, the parotid gland of NOD mice, with constitutive isoforms involved.

The observations described above suggested that submandibular glands from NOD mice presented a defective NOS, and this could be explained by either an altered expression of one or more isoforms or an almost complete down-regulation of constitutive isoforms as, for instance, that reversed by PKC inhibitors reported previously in another model (23). In this regard, it is interesting to note that PKC-sensitive NOS present in the submandibular glands referred to above appeared to be a neural isoform that displayed lower basal and stimulated activity than it did in other tissues (23), but not the absence of NOS-specific activity as that observed in NOD submandibular glands reported here. On the basis that both neural and endothelial NOS (NOS I and III) are expressed in submandibular glands and that the "inducible" NOS II isoform is also constitutively expressed in some mammalian tissues, including these glands (16, 26, 27), we decided to investigate whether any of the three isoforms of NOS was abnormally expressed in NOD submandibular glands. The results argue for differential expression of NOS in NOD submandibular glands, since there were clear differences in the pattern of one isoform, the neural NOS, and no changes could be stated in the expression of the other two. Though we cannot rule out that a posttranslational down-regulatory effect was affecting NOS activity, the altered expression of NOS I in NOD submandibular glands assessed by immunobloting suggests that mechanisms affecting the integrity and/or stability of this isoform at other transcriptional and posttranscriptional levels may also be taking place in this model. Altered synthesis and secretion of several salivary proteins has already been reported in NOD mice, including diminished amylase and EGF concentrations and an aberrant expression of PSP in submandibular glands (28, 29). Based on those results and on studies of PSP mRNA and protein expression in parotid glands of normal neonate mice, it has been hypothesized that changes in protein expression may be due to a rapid turnover of PSP transcripts in the cytoplasm or to the activity of the appropriate cytoplasmic factors for processing and posttranslational modifications (28–30). In order to see whether the effect on NOS I was a hallmark of submandibular glands or a more general effect that affected other salivary glands and tissues, we tested the expression of the three isoforms in parotid glands, brain, and spleen of NOD and control mice. Interestingly, altered expression of NOS I was also found in parotid glands but not in cerebral cortex and spleen of NOD and BALB/c mice. The fact that different salivary glands of NOD mice presented altered expressions of NOS I with either a lower molecular weight or a lower expression of the corresponding band, but in both cases displaying no significant specific activity, strongly supports the need of both assays—activity and expression—run in parallel to assess an altered function of this enzyme in one tissue.

Regarding the expression of the other two isoforms, both NOS II and III were similarly expressed in NOD and BALB/c mice in salivary and nonsalivary tissues tested. Again, though NOS III was apparently normally expressed in NOD with respect to controls, it seemed not to be active, as shown in activity assays with EGTA, in either NOD glands or BALB/c. Certainly, we have recently shown by pharmacological means the predominance of neural rather than endothelial origin of constitutive NOS activity in normal submandibular and parotid glands (23, 31). Thus, the

altered expression of NOS I shown here appeared not only specific for submandibular and parotid glands but also for this isoform, an interesting result considering the proposed functional/neural origin of the secretory dysfunction found in NOD mice and in Sjögren's patients discussed earlier. The fact that the alteration of NOS expression involved only one isoform, likely the neural isoform, seems attractive not just for this mouse model of autoimmune sialadenitis or the sicca syndrome accompanying other autoimmune rheumatic diseases, but also for other nonimmune diseases that course with xerostomia, most of them secondary to chronic drug treatments. NOS expression in labial minor salivary glands of Sjögren's patients has been studied previously (26). By means of immunocytochemical analysis of these glands, the authors reported on a very sparse distribution of NOS I-containing nerve fibers, mainly localized around acini and ducts with no immunoreactive fibers in inflammatory cell foci, and parallel changes in NOS III expression, but the endothelial isoform did 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 (26). These results are in accordance with those described here for NOD mice and point to the potential role of impaired neurotransmitter receptor signaling in the ulterior salivary dysfunction.

Finally, the altered expression and function of NOS I in submandibular and parotid glands described in this study occurred with a concomitant decrease in salivary flow rate, as found in NOD mice stimulated with pilocarpine plus VIP compared to controls; the effect was even higher than that with pilocarpine alone in these mice, suggesting a progressive loss of salivary gland autonomic function. In support of this, VIP-stimulated amylase and protein concentrations in saliva of NOD mice were not significantly decreased with respect to control mice, confirming that different signaling pathways mediate the effect of VIP on either watery or proteinaceous secretions. We cannot rule out that, in addition to VIP's direct effect in this model of sialadenitis, it could also exert an indirect influence on secretion by influencing the release of autacoids, considering its role as a scavenger of reactive oxygen radicals (32). These observations are similar to those previously reported by other groups working with prediabetic NOD mice in which decreased levels of saliva production were found in response to muscarinic plus VIP receptor activation (33), although, in our hands VIP-mediated salivary flow was by far more affected than muscarinic secretion in submandibular glands of NOD mice. The possibility that a preexisting defect in hormone-receptor signaling in the target organ preceded structural damage in the development of an autoimmune response has already been discussed for the Obese strain chicken model of spontaneous autoimmune thyroiditis (34). Similarly, we have shown previously, in an experimental model of autoimmune myocarditis, that neurotransmitter-stimulated contractile dysfunction preceded histological lesions of cardiac tissue (20). Although additional studies are required to demonstrate whether this is the case in autoimmune sialadenitis, the fact that NOS from neural origin was altered in submandibular and parotid glands of an experimental model of sialadenitis (with only incipient signs of xerostomia, as reported here) and that nitric oxide-mediated signaling has a role in saliva secretion strongly suggest that such alteration of NOS I, with the subsequent impairment of neurotransmitter signaling, might be one of the earliest changes to occur in the gradual loss of saliva secretion. Evidence of reduced responses to either β-adrenergic or muscarinic acetylcholine receptor activation has been presented in salivary glands of NOD mice (12, 35); however, the mechanisms underlying these alterations remain to be elucidated. Certainly, it has been hypothesized that serum autoantibodies from primary Sjögren's syndrome patients could promote abnormal signaling in salivary and lacrimal glands by interacting with neurotransmitter receptors (6–8), and similar observations have been reported for NOD mice (36), as well as for other human and murine autoimmune responses (37, 38). In addition to antibodies, cytokines found in salivary glands of patients (3) and models (11) are also good candidates for mediating some of the changes referred to above, either directly or through the activation of common signaling cascades and cross talk (reviewed in 39). The possibility that neuropeptide levels in affected organs were modified has also been analyzed elsewhere (33). Nevertheless, as reported for other autoimmune responses and autoimmune-based disorders, the symptoms and signs observed are usually the result of a complex series of events, which might even include more than one of the hypotheses proposed above. Therefore, the challenge is to find suitable experimental approaches that allow us to gain more insight into the intracellular mechanisms underlying autoimmune diseases and shed light on ways by which autoimmune damage can be reduced.

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