Role of nitric oxide in Sjogren's syndrome

Konttinen, Y T

1997


http://hdl.handle.net/10138/33780

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.
ROLE OF NITRIC OXIDE IN SJÖGREN’S SYNDROME

YRJÖ T. KONTTINEN, LOUISE A. M. PLATTS, SUSANNE TUOMINEN, KARI K. EKLUND, NINA SANTAVIRTA, JYRKI TÖRNWALL, TIM0 SORSAS, MIKA HUKKANEN, and JULIA M. POLAK

Objective. To measure levels of salivary nitrite (NO$_2^-$) and to localize nitric oxide synthases (NOS) in the labial salivary glands (LSGs) of patients with Sjögren’s syndrome (SS).

Methods. NO$_2^-$ was measured by the Griess reaction. LSGs were analyzed using NADPH-diaphorase histochemical and immunohistochemical studies to determine the constitutive NOS (neuronal [ncNOS] and endothelial [ecNOS]) and inducible NOS (iNOS) isoforms.

Results. The NO$_2^-$ concentration (mean ± SEM 307 ± 51 μM versus 97 ± 16 μM; P < 0.05) and output (166 ± 46 nmmoles/minute versus 37 ± 7 nmmoles/minute) were increased in SS patients compared with healthy control subjects. NADPH-diaphorase was found in some nerve fibers and endothelial cells, and, in SS, was found in myoepithelial, acinar, and ductal epithelial cells, but in only a few inflammatory cells. In SS, ncNOS-immunoreactive nerve fibers were sparse and ecNOS was found in a minority of the CD31-positive vascular endothelial cells and acinar cells, whereas iNOS was localized in myoepithelial, acinar, and ductal epithelial cells, often together with tumor necrosis factor α.

Conclusion. Nitrite was found in normal human saliva. NO produced by ncNOS probably acts as a non-adrenergic, noncholinergic neurotransmitter, whereas that produced by ecNOS exerts a vasodilatory effect. SS patients had increased NO$_2^-$ concentrations, with most of the superfluous salivary NO being produced not by the immigrant inflammatory cells, but rather, by the resident salivary gland cells. NO may contribute to inflammatory damage and acinar cell atrophy in SS.

Nitric oxide (NO) is produced by vascular endothelium, nerve terminals, and inflammatory cells in a reaction catalyzed by NO synthase (NOS), which converts l-arginine to NO and l-citrulline (1). NOS occurs in at least 3 different isoforms, endothelial constitutive NOS (ecNOS), neuronal constitutive NOS (ncNOS), and inducible NOS (iNOS) (2,3). Both ecNOS and ncNOS are constitutively expressed, but are regulated as to their level of activity by various soluble ligands and/or shear forces through calcium/calmodulin-dependent second messenger pathways. NO concentrations produced by this route are usually relatively low (in the picomolar range). In contrast, regulation of iNOS occurs at the level of gene transcription and is relatively slow, but leads to long-lasting and significant increases in NO production (giving rise to nanomolar local concentrations of NO) (1,3,4).

NO has a short half-life and is rapidly converted to nitrites and nitrates. Therefore, its range of action is limited. This is important from the point of view of regulation, because NO itself is a small, lipid-soluble compound, which easily penetrates the cell membrane of nearby cells and can therefore affect them in a receptor-independent autocrine and/or paracrine manner. At the same time, NO is a highly reactive compound, due to an unpaired electron in its molecular orbital. At low concentrations, it preferentially activates guanylate cyclase, which thus produces cGMP. At high concentrations, NO inhibits various iron-containing DNA synthetases and mitochondrial enzymes, and thus inhibits cell growth and division, finally leading to cell death (5).

Due to its potential dual role in normal salivary gland function and damage in pathologic conditions, it was considered important to characterize the eventual
presence of NO in saliva and to localize the cells/NOS isoforms responsible for NO production in patients with Sjögren's syndrome (SS).

PATIENTS AND METHODS

Patients and samples. All patients and healthy volunteers gave their informed consent. The Declaration of Helsinki (6) was followed throughout the study, which was approved by the local ethics committee.

Both patients and controls provided a stimulated salivary sample, as described elsewhere (7). Briefly, stimulation of saliva was achieved by the chewing of paraffin for 5 minutes, and the stimulated whole saliva was then collected. Immediately after collecting the saliva, the samples for NO2− measurement were centrifuged at 1,000g for 5 minutes, and the supernatants were frozen at −70°C until analyzed. For vasoactive intestinal peptide (VIP) measurement, the saliva was collected into chilled tubes containing 50,000 IU/ml aprotinin and 10 mM EDTA to minimize artificial neuropeptide degradation in vitro (8).

The mean age of the 12 primary SS patients, who were all women, was 51.6 years (range 21–74). The duration of the SS was a mean of 9 years (range 1–23). All patients were nonsmokers. The mean age of the 10 healthy control women was 47.7 years (range 39–56).

The dental/periodontal features of all patients were evaluated for decayed, missing, or filled surfaces using the DMFS index (9), bleeding on probing using the BOP index (10), presence of visible plaque using the visible plaque index (11), retentive calculus (subgingival calculus) using the RC index (12), items on the community periodontal index of treatment needs (13), and pocket probing depth (14). Eventual correlations were sought between the dental and periodontal status and salivary NO2− concentration.

Between 6 and 8 labial salivary glands (LSGs) were obtained from each patient by biopsy. Samples were taken by blunt dissection under infiltration anesthesia and fixed by immersion in freshly made 1% paraformaldehyde in 0.01M phosphate buffered 0.15M saline (PBS; pH 7.1) at 4°C for 16–18 hours. After fixation, cryoprotection was performed in 16–18 hours. After fixation, cryoprotection was performed in 30% (weight/volume) sucrose and 0.01% (w/v) sodium azide in PBS for several days at 4°C before the samples were embedded in OCT compound (Lab-Tek Products, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −70°C until staining. LSGs were obtained from 4 patients with SS who fulfilled the European criteria for SS (14), and from 2 healthy controls who did not have SS (14) and who had a focus score of <1 (15).

Jenkins activity survey (JAS). The JAS scale consists of 84 items, which are divided into 4 subscales. For the present study, we recorded the values of the actual A-B factor, which involves 31 items on the JAS that best reflect the stress behavior of a person (16). The reliability of the stress factor was confirmed with the use of the Cronbach α test (17).

Acetylcholinesterase histochemical analysis. Acetylcholinesterase activity in LSG tissue was visualized as previously described (20). Briefly, the cryostat sections were first immersed in an incubation medium for 45 minutes at 37°C. The acetylcholinesterase activity was then visualized by immersion of the sections for 5 minutes in 50 mM Tris HCl buffer, pH 7.6, containing 0.04% 3,3′-diaminobenzidine and 0.3% nickel ammonium sulfate. Sections were incubated in this solution for a further 5 minutes at addition of hydrogen peroxide, for a final concentration of 0.003%.

Immunocytochemical analysis for NOS. Polyclonal antibodies for the cNOS and iNOS isoforms used in this study have been characterized in detail elsewhere. Briefly, antibodies for ncNOS, kindly provided by Drs. S. Moncada and V. Riveros-Moreno (Wellcome Research Laboratories, Beckenham, Kent, UK), were raised against the whole protein purified from rat brain and against synthetic peptide fragment 53 of rat brain ncNOS; both of these antiserum cross-react with the ecNOS isoform at high concentrations (21). Monoclonal antibodies for ecNOS were raised against a purified whole protein of particulate bovine aortic endothelial NOS isoform (22) and were kindly provided by Dr. J. S. Pollock (Abbott Laboratories, Abbott Park, IL). Antibodies for iNOS were raised against synthetic peptides corresponding to amino acid residues 47–71 of murine macrophage iNOS and 54–76 of human hepatocyte iNOS (23–25). Murine macrophage iNOS antibodies were kindly provided by Drs. S. Moncada and V. Riveros-Moreno (Wellcome Research Laboratories) and human hepatocyte iNOS antibodies by Dr. T. J. Evans (Royal Postgraduate Medical School).

Antibodies against the synthetic 47–71 peptide of macrophage iNOS have been characterized for specificity in detail elsewhere, with the use of an antigen preabsorption test and Western blotting (25). Similarly, the specificity of the human hepatocyte iNOS antibodies have been confirmed by antigen reabsorption test with the use of a synthetic 54–76 peptide, by Western blotting, and by comparison with in situ
Table 1. Correlations between the salivary nitrite (NO$_2^-$) concentration and dental and periodontal status of 10 patients with Sjögren's syndrome*

<table>
<thead>
<tr>
<th>Dental/periodontal status</th>
<th>Spearman's rank correlation coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMDFS</td>
<td>0.2492</td>
<td>0.4874</td>
</tr>
<tr>
<td>BOP</td>
<td>0.5294</td>
<td>0.1155</td>
</tr>
<tr>
<td>VPI</td>
<td>-0.2339</td>
<td>0.5155</td>
</tr>
<tr>
<td>RC</td>
<td>-0.4786</td>
<td>0.1617</td>
</tr>
<tr>
<td>PPD (4-5 mm)</td>
<td>-0.3556</td>
<td>0.3133</td>
</tr>
<tr>
<td>PPD (&gt;6 mm)</td>
<td>-0.0580</td>
<td>0.8735</td>
</tr>
</tbody>
</table>

* DMDFS = decayed, missing, or filled surfaces; BOP = bleeding on probing; VPI = visible plaque index (yes/no); RC = retentive calculus (subgingival calculus); PPD = pocket probing depth. The community periodontal index of treatment need values were zero for all patients studied. None of the correlations were statistically significant.

Radioimmunoassay for VIP. The neuropeptide concentration in saliva was measured using a standard competitive 125I-radioimmunoassay kit (Peninsula, Belmont, CA) as described in detail elsewhere (8). Briefly, 1) 100 μl of sample or standard was incubated in polypropylene tubes for 24 hours at 4°C with 100 μl of rabbit anti-human VIP IgG, 2) 100 μl of 125I-labeled peptide (10,000–15,000 counts per minute/100 μl) was added and incubated for 24 hours at 4°C; 3) 100 μl of goat anti-rabbit IgG and 100 μl of normal rabbit serum were added and incubated for 90 minutes at room temperature before 4) 500 μl of radioimmunoassay buffer was added, followed by centrifugation at 1,700g for 20 minutes at 4°C. The supernatant was discarded and the radioactivity of the pellet was measured with a programmed gamma scintillation counter. All samples were analyzed in duplicate, with individual controls for apparent binding in the absence of antibody (i.e., total counts, nonspecific binding, total binding).

Statistical analysis. Results are presented as the mean ± SEM. Comparisons between the 2 groups were performed with the Student’s t-test for normally distributed variables, and with the Mann-Whitney U test for non-normally distributed variables. Analysis of the linear relationship between 2 variables, Pearson’s correlation coefficient was used for normally distributed variables and Spearman’s rank correlation coefficient for non-normally distributed variables.
Figure 1. Histochemical and immunohistochemical localization of NADPH-diaphorase activity and the 3 different isoforms of nitric oxide synthase (NOS) in labial salivary glands from patients with Sjögren's syndrome. A, Histochemical demonstration of NADPH-diaphorase activity within myoepithelial cells (arrows) and acinar cells of acini (open arrow) and excretory duct epithelia (arrowheads). B, Neuronal NOS immunoreactivity in nerve fibers (arrows) terminating near an acinus. Neuronal NOS–containing nerve fibers can also been seen in connective tissue surrounding the acinus. C, CD31-positive vascular endothelial cells (arrows) within a small focal inflammatory cell infiltrate surrounding an excretory duct. D, Endothelial NOS (arrows) was found to colocalize with CD31-positive cells, as evidenced by immunostaining of serial sections. E, Immunoreactivity for endothelial NOS in several acini localized mainly in acinar cells (arrows). F, Antibodies for human hepatocyte inducible NOS (iNOS) isoform reveal numerous iNOS-immunoreactive mononuclear cells (open arrows) within an inflammatory cell infiltrate. (Original magnification × 400.)
except for those in the JAS, which were analyzed using the SPSS software package (SPSS Inc., Chicago, IL).

RESULTS

Relationship of salivary NO$_2^-$ to SS, stress, and VIP. The NO$_2^-$ concentration was higher in patients with SS (mean $\pm$ SEM 307 $\pm$ 51 $\mu$M) than in healthy control individuals (97 $\pm$ 16 $\mu$M; $P < 0.05$). There was no correlation between the salivary NO$_2^-$ concentration and the dental or periodontal status of the SS patients (Table 1). The NO$_2^-$ output was 37.2 $\pm$ 6.6 nmoles/minute in healthy controls compared with 166.3 $\pm$ 46.2 nmoles/minute in patients with SS ($P < 0.01$). The salivary VIP concentration was 68.6 $\pm$ 24.2 pg/ml in the patient group compared with 40.8 $\pm$ 23.6 pg/ml in the healthy control group ($P < 0.05$). However, there were no significant correlations between salivary NO$_2^-$ and VIP concentrations or between salivary NO$_2^-$ concentration and stress in the patients or in the controls.

NADPH-diaphorase activity in LSGs in SS. The most conspicuous finding was that, in the LSGs, intense NADPH-diaphorase activity was localized in myoepithelial cells of the glandular acini and less frequently in acinar end-piece cells (Figure 1A). Epithelial cells of intercalated, striated, and excretory ducts also showed intense NADPH-diaphorase activity (Figure 1A). It was particularly interesting that such epithelial-cell NADPH-diaphorase expression was also seen in the LSGs obtained from healthy controls. NADPH-diaphorase activity was found in some nerve trunks, fibers, and terminals located around the acini and salivary ducts. Such NADPH-diaphorase-containing nerve fibers and terminals were relatively evenly distributed, but seemed to be absent from lymphocyte-infiltrated areas (i.e., lymphocyte foci) in SS patients.

NADPH-diaphorase activity was less frequently localized in endothelial cells of arterioles, capillaries, or venules. NADPH-diaphorase–positive endothelial cells were, in particular, observed in lymphocyte infiltrates in SS patients (for more details, see below). NADPH-diaphorase activity was seen in only a few of the inflammatory cells of the characteristic focal lymphocyte-rich foci (for more details, see below).

Neuronal eNOS in LSGs in SS. Antibodies raised against the whole nNOS protein purified from rat brain and the synthetic peptide fragment 53 of rat brain nNOS showed a very sparse distribution of nNOS-containing nerve fibers, mainly localized around the acini and along the salivary ducts (Figure 1B). However, nNOS immunoreactive nerve fibers were not seen in inflammatory cell foci in SS. In contrast, acetylcholinesterase histochemical and immunocytocchemical localization of VIP-containing nerve fibers revealed a very dense distribution of cholinergic and VIPergic (parasympathetic) nerve fibers coiling around the acini and salivary ducts in noninflamed areas of LSG, although such fibers appeared to be mostly absent from the lymphocyte foci in SS (data not shown).

Endothelial eNOS in LSGs in SS. Monoclonal antibodies raised against the bovine aortic ecNOS isoform revealed a very sparse endothelial cell ecNOS immunoreactivity compared with immunoreactivity for CD31. However, it was of interest to note that in SS, CD31-positive endothelial cells in focal inflammatory cell infiltrates showed a strong immunoreactivity for ecNOS (Figures 1C and D). Some ducetal epithelial cells showed dense granular immunoreactivity and many acini were also immunoreactive for this antiserum (Figure 1E).

Inducible NOS in LSGs in SS. Antibodies raised against both the murine macrophage and human hepatocyte iNOS peptide sequences were used in the present study. The 2 antibodies gave somewhat different profiles of iNOS-immunoreactive cells in inflammatory LSG tissue. Murine macrophage iNOS antiserum showed immunoreactivity for acinar structures and some inflammatory cells, whereas human hepatocyte iNOS antibodies showed more frequent immunoreactivity for mononuclear inflammatory cells (Figure 1F) as well as for some ducetal epithelial cells (data not shown).

In SS patients, granular TNF$\alpha$ staining was seen in some acini, vascular endothelial cells, and, most notably, ducetal epithelial cells. Immunostaining of serial sections for TNF$\alpha$ and human hepatocyte iNOS peptide sequence showed colocalization of TNF$\alpha$ with iNOS in ductal epithelial cells, but not in acinar or vascular endothelial cells (Figures 2A and B).

The chemical reaction of NO with superoxide generates peroxynitrate, which has been shown to cause nitration of protein tyrosine groups. Antibodies against nitrosylated tyrosine (nitrotyrosine) residues labeled some ductal epithelial cells, but mainly detected nitrotyrosine in periductal connective tissue. Periacinar connective tissue was also, in some cases, nitrotyrosine positive, but nitrotyrosine immunoreactivity could not be detected within the acinar cells (data not shown). Nitrotyrosine-staining controls were negative.

Cell counting showed human hepatocyte iNOS in some immigrant inflammatory cells in the lymphocyte-rich foci in patients with SS. Approximately 16 $\pm$ 3 (mean $\pm$ SEM) such cells were seen per 1 mm$^2$ of
inflammatory cells, compared with 21 ± 2 CD68+ macrophages. However, colocalization studies with double labeling suggested that there was only a minor overlap between these 2 cell subpopulations (data not shown), although some CD68-positive macrophages were also immunoreactive for human hepatocyte iNOS (Figures 3A and B).

**DISCUSSION**

SS is characterized by a focal adenitis, autoimmunity, and sicca symptoms. In salivary glands, this is expressed in the form of focal T lymphocyte-rich infiltrates and xerostomia. The simplistic and prevailing view has been that sialopenia and xerostomia are due to structural destruction of the secretory acinar cells, which are responsible for the secretion of the primary saliva. However, there is a clear discrepancy between the often focal and local salivary gland involvement and the greatly diminished salivary flow seen in SS. Significantly, many patients draw advantage from the use of sialogogues, such as xylitol gum or pilocarpine. Therefore, an alternative hypothesis has been that, before permanent

---

**Figure 2.** Immunoreactivity for human inducible nitric oxide synthase (iNOS) antibodies (A) (arrows) was found to colocalize with that for tumor necrosis factor α (TNFα) (B) (arrows) in ductal epithelial cells in labial salivary glands from patients with Sjögren’s syndrome. The 2 sections immunostained for iNOS and TNFα were taken 40 μm apart, but several ducts can still be traced through the tissues. Note the absence of iNOS and TNFα immunoreactivity in acini. (Original magnification × 400.)

**Figure 3.** Indirect immunofluorescence technique was used for colocalization of inducible nitric oxide synthase (iNOS) immunoreactivity (A) (arrows) with CD68-positive macrophages (B) (arrows). In these photomicrographs, only 2 CD68-positive macrophages (B) also express iNOS immunoreactivity, although some additional inflammatory and/or resident cells were immunoreactive for the iNOS isoform (A). (Original magnification × 450.)
salivary gland parenchymal damage has occurred in a long-lasting and chronic disease, the focal perivascular and periductal inflammation alters the vasoneural regulation of the various vascular, motor, and secretory components of the normal salivary flow.

Using VIP and neuropeptide Y as markers for postganglionic parasympathetic and sympathetic nerve fibers, respectively, a clearly decreased innervation of the salivary glands in SS has been shown (28,29). In addition to adrenergic and cholinergergic nerves, the non-adrenergic, noncholinergic system plays a role in normal and abnormal neuronal regulation of the salivary glands. An important part of the nonadrenergic, noncholinergic system is mediated by VIP, which, via its low-affinity binding sites, is linked to cAMP formation and, via its high-affinity binding sites, to Ca²⁺ mobilization and promotion of neuronal survival (30–32). More recently, NO has been measured in human saliva (33). It was also confirmed that NO is released from salivary glands rather than being produced from the activity of oral bacteria (33). Furthermore, there were no significant correlations between the salivary NO₂⁻ concentration and the periodontal status of the SS patients, which rules out any contribution of inflammatory gingival crevicular fluid to the salivary NO₂⁻. This is consistent with recent studies on the presence of NADPH-diaphorase activity and NOS isoforms in feline submandibular glands (34) and with the current findings on the presence of NADPH-diaphorase activity and of all 3 different NOS isotypes in human salivary glands.

Formation of primary saliva involves vasodilation and acinar cell secretion. Changes in the regulatory vasoneural interactions may lead to sialopenia and dry mouth, such as is seen in psychogenic dry mouth in rather than being produced from the activity of oral bacteria (33). Changes in the L-arginine/NO-generating pathways are also found in various cytokines and lipopolysaccharides. In particular, certain cytokine combinations, such as interferon-γ (IFNγ), interleukin-1 (IL-1), and TNFα, are very effective and synergistic in the up-regulation of iNOS gene expression (40,41). Inducible NOS produces high concentrations of NO for extended time periods, and, often, high cytotoxic and bactericidal concentrations of NO are produced.

Increased NO production hardly represents attempts to compensate for diminished cholinergic stimulation. It seems that the increased NO production is caused by local inflammatory changes and by cytokines, and, as such, appears to be an effect of inflammation rather than of a physiologic process intended to compensate for diminished cholinergic stimulation. Moreover, increased inflammatory NO production mediated by iNOS might not be able to compensate for the highly regulated neuronal events that are involved in the coordinated vascular, motor, and secretory components of the reflex salivary flow. Inflammatory NO production would be very unlikely to have that type of effect,
coordinated in time and at site, that is characteristic of a well-functioning normal neurovascular system of the salivary gland.

It was of particular interest to note that NADPH-diaphorase activity and, to a lesser extent, iNOS expression, were seen in the acinar and ductal epithelial cells of LSGs from SS patients, but also to some extent in those from healthy controls. In thyroid glands, NADPH-diaphorase activity and NOS immunoreactivity have been described in nerve fibers and vascular endothelium, but not in the glandular parenchymal cells (42). Lohinai and coworkers, in a study of feline submandibular glands, noticed that the epithelial layer of the salivary ductal branches was NOS immunonegative, but NADPH-diaphorase positive (34). Inducible NOS/NADPH-diaphorase in salivary glands might be induced by bacterial lipopolysaccharides (40). However, considering the cytokine-mediated regulation of iNOS, it seems more likely that in SS, iNOS immunoreactivity and NADPH-diaphorase activity are induced by cytokines, such as TNFα, IL-1, and IFNγ (40,41), which are produced by the salivary gland epithelial cells and/or activated T lymphocytes (43–45).

One interesting feature of the minor salivary glands is the occurrence of focal accumulations of lymphocytes around the duct walls (46–48), which are believed to have a role in the immune surveillance of the mouth (48). Although the control LSGs were from subjects who did not have SS and who had a focus score (10) of <1, they did have occasional lymphocyte foci. These foci might produce enough cytokines (43–45) to up-regulate the iNOS immunoreactivity/NADPH-diaphorase activity even in normal minor salivary glands, although not to the same extent as in SS, as suggested by the present findings on salivary NO₂⁻ concentration.

The salivary concentration of NO₂⁻ may not quite reflect the extent of local NO production, because at least part of the locally produced NO could be disposed via pathways other than that leading to saliva. It is therefore possible that the increased, inflammation-associated, and probably cytokine-induced local NO production in SS interferes with the normal function of the eNOS isoforms and may, by itself, lead to cytopathic changes and acinar cell atrophy.

Thus, NO is produced in physiologic amounts in normal salivary glands mainly by constitutively expressed nNOS and eNOS isoforms, and may contribute to various events that regulate vascular reactivity, salivary flow, and neuropeptide release. In focal adenitis in SS, cytokine-induced iNOS is expressed by salivary gland acinar and ductal epithelial cells together with TNFα, suggesting a role for these resident cells in local pathomechanisms, which may contribute to various cytopathic and atrophic cell changes.

REFERENCES
6. Declaration of Helsinki, Hong Kong amendment, September 1989
20. Wharton J, Polak JM, Gordon L, Banner NR, Springall DR, Rose
NITRIC OXIDE IN SS


