Control of Salivary Secretion by Nitric Oxide and Its Role in Neuroimmunomodulation

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ABSTRACT: **In many *in vivo* systems exposure to endotoxins (LPS) leads to the co-induction of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which is important to the regulation of the function of different systems during infection. In submandibular glands (SMG) neural (n)NOS is localized in neural terminals and in striated, granular convoluted and excretory ducts, endothelial (e)NOS in vascular endothelium and ducts, and iNOS in macrophages and in tubules and ducts. In normal adult male rats, injection of an inhibitor of NOS decreased the stimulated salivary secretion and a donor of NO potentiated it, indicating that NO exerts a stimulatory role. A single high dose of LPS (5 mg/kg, i.p.) induced an increase in NOS activity measured by the ^{14}C-citrulline method, increased PGE content almost 100% as measured by RIA, and blocked stimulated salivary secretion. The administration of a specific iNOS inhibitor, aminoguanidine (AG), with LPS not only decreased NOS activity but significantly decreased PGE content, indicating that NO triggered the activation of COX-2. LPS increased conversion of labeled arachidonate to prostaglandins (PGs) showing that COX was induced. Since a PGE_1 analogue blocked stimulated salivation, the LPS-induced inhibition of salivation is probably due to release of PGs. Therefore, the use of inhibitors of iNOS and COX-2 could be very useful to increase salivation during infection since saliva has antimicrobial actions.**

INTRODUCTION

The submandibular gland is one of the major salivary glands together with the parotid and sublingual glands. The initial saliva is secreted by acini into the terminal end of the ducts and drains into the intercalated ducts that are succeeded by granular convoluted tubules (GCT). The cells in GCTs are characterized by numerous serous-type secretory granules in their cytoplasm. These granules are the repositories of a variety of bioactive substances, including both nerve growth factor and epidermal growth factor and the peptidases, kallikrein and renin. In the rat these GCTs are

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succeeded by striated ducts, the appearance of which is due to vertically oriented mitochondria alternating with highly folded plasma membranes. In the rat the principal cells of these ducts are filled with granules. Finally the striated ducts empty into the excretory ducts.[1]

The secretion of saliva is controlled by the autonomic nervous system. The parasympathetic nervous system is the main controller of this secretion via impulses in the chorda tympani nerve that innervates it and releases acetylcholine and substance P (SP). Both can evoke copious salivary secretion by activating muscarinic and tachykinin-1 receptors, respectively. The sympathetic nervous system also controls salivary secretion by acting on α- and β-adrenergic receptors.

Nitric oxide (NO) controls the function of many organs of the body. Nitric oxide synthase (NOS) is present in nerve terminals that are widely distributed in various parts of the submandibular gland (SMG). Therefore, we hypothesized that NO would play a role in control of salivary secretion. Indeed, we found that inhibition of NOS activity by L-nitro-arginine-methyl-ester (L-NAME) or monomethyl-L-arginine (L-NMMA) injected i.v. to rats prior to stimulation of salivary secretion with methacholine (MC) or SP inhibited significantly the secretion of saliva.[2] The SMG contains all three isoforms of NOS: calcium-dependent neural (n)NOS and endothelial (e)NOS and also a calcium-independent NOS (iNOS) as visualized by immunohistochemistry.[2]

Since the salivary glands are a major portal of entry of bacteria and viruses into the body, we wished to evaluate the effects of infection, as mimicked by injection of bacterial lipopolysaccharides (LPS), on salivary secretion and on the activity of NOS in the SMG. It is well known that prostaglandins also play a very important role in inflammation and are induced by NO, which activates cyclooxygenase (COX). Therefore, we also evaluated the role of PGs in the response of the gland to LPS. LPS was found to decrease salivary secretion in response to secretogogues. Since saliva contains a number of substances involved in mediating the defense of the body against pathogens entering the mouth, the decrease in salivary secretion during endotoxemia could be detrimental.

MATERIAL AND METHODS

Salivary Secretion Studies

Male Wistar rats (250–300 g) were used and were housed under standard conditions (12h light/12h dark cycle, at 22–25°C) and with free access to rat chow and tap water. Food was removed 14 h prior to experimental procedures in order to decrease variation in salivary secretion. Salivary responses were determined in anesthetized rats (chloralose 100 mg/kg i.v.). The ducts of the SMG were cannulated and dose-response curves were obtained through the sequential injection, via the right femoral vein, of increasing doses of various secretagogues as described previously.[3]

In order to investigate the role of NO on saliva secretion, rats were injected i.v. with NOS inhibitors, L-NAME or L-NMMA, 1 h prior to injection of increasing doses of sialogogue, to calculate the same dose-response curves in the presence of one of the NOS inhibitors. In another series of experiments one of the sialogogues was injected at the same dose every 5 min. MC was injected at a dose of 10 μg/kg and

noradrenaline (NA) at a dose of 30 μg/kg. In order to evaluate whether NO donors would increase the salivary secretion, nitroglycerin was injected (100 mg/kg) i.v. alone or just prior to injection of the second pulse of MC or NA. All the results are expressed as mean volume of saliva secreted ± SEM (assuming saliva density of 1.0 g/ml).

Determination of NOS Activity

A modification of the method of Bredt and Snyder that measures the conversion of [^{14}C]arginine into [^{14}C]citrulline was used.[4] The method indirectly measures NO production, an index of NOS activity. Since the SMG has an active urea cycle in the tissue, arginine will also be converted to citrulline by this cycle, thereby giving false high values for NOS activity. This problem was obviated by addition of L-valine (25 mM) to the HEPES buffer for homogenization and incubation that blocks the arginase of the urea cycle. The details of the method used were published previously.[2]

Measurements of PGE Content in SMG

PGE content of the SMG was measured by specific radioimmunoassay as a determinant of COX activity as described elsewhere.[5]

Measurements of Radioconversion of [^{14}C] Arachidonic Acid (AA) to Prostanoids in SMG

This was performed by chromatography of ethylacetate extracts and counting of the labeled prostanoids as previously reported.[6] The area of each of the radioactive peaks corresponding to authentic prostanoids was calculated and expressed as a percentage of the total radioactivity of the plates.

Measurements of Plasma Nitrite (NO_2)/Nitrate (NO_3)

NO_2/NO_3 serum concentration (as a marker for endogenous NO production) was determined by colorimetric assay with a nitrate/nitrite assay kit (Cayman Chemical Co., Cat. # 780001, Ann Arbor, MI 48108).

Immunohistochemical Localization of NOS

The antisera used in this study were raised in rabbits against different NOS isoenzymes and immunocytochemical studies were performed by the method of Julia Polak.[7]

RESULTS

Distribution of NOS in SMG

nNOS immunoreactivity was demonstrated in several segments of the duct system of the SMG but was absent from the acini. This activity was massive when we compare it to NOS activity of nerve fibers (nNOS) or macrophages (iNOS) in the gland. Within the ducts, we could differentiate between a membrane-associated and

a cytoplasmic NOS immunoreactivity. Membrane-associated NOS was greater for iNOS than nNOS (see FIGURES 1 and 2 and TABLE 1). eNOS was not studied but has been localized to vascular endothelium and salivary ducts.[8]

Effect of NO on Salivary Secretion

Since there is little or no spontaneous salivary secretion in the rat in our conditions, salivary secretion could only be studied after injection of various secretogogues. Prior injection of L-NAME or L-NMMA, (as described above) inhibited significantly the response to MC or SP.[2] In the present study we evaluated the response to isoproterenol (a β-adrenergic receptor agonist) and NA before and after the injection of L-NMMA. There was a decrease in salivary secretion after inhibition of NOS which became significant with the highest dose of isoprotenerol or NA (see FIGURE 3).

In order to determine if NO itself stimulates salivary secretion, we injected a NO donor, nitroglycerin (100 mg/kg) i.v. The injection of nitroglycerin alone had no significant effect on salivary secretion. Therefore, we used MC or NA as sialogogues. MC was injected at the high dose (10 μg/kg) every 5 min during 20 minutes. Nitroglycerin (100 mg/kg) was injected one minute before the second injection of MC. There was a significant potentiation of salivary outflow after administration of MC

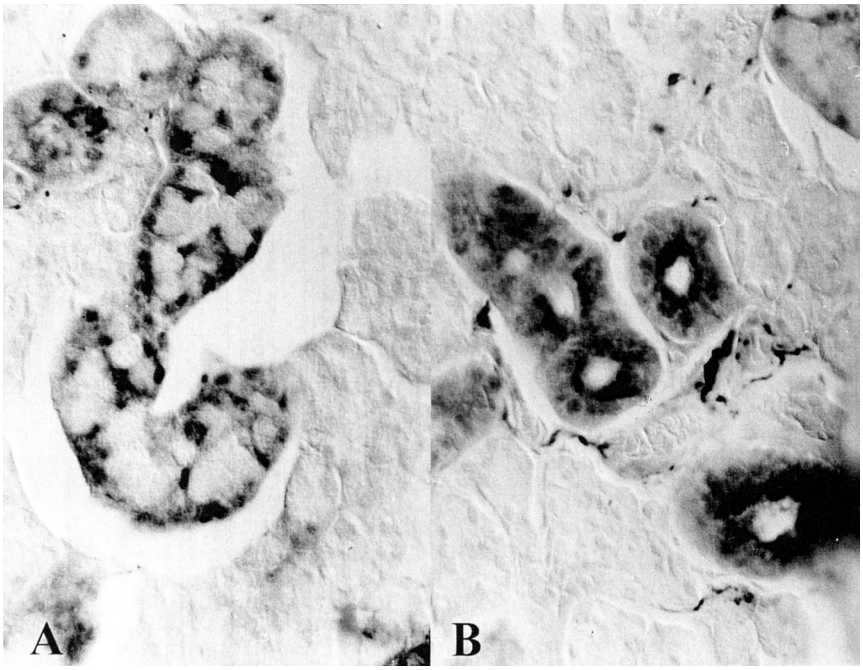

FIGURE 1. Immunohistochemical localization of nNOS in SMG. **A:** a granular convoluted tubule and **B:** striated ducts (1200×).

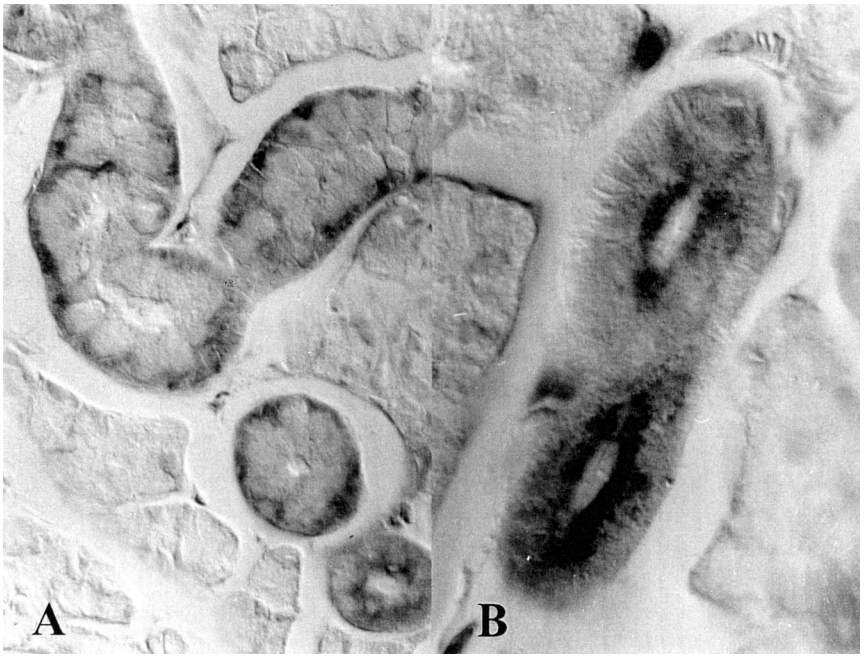

FIGURE 2. Immunohistochemical localization of iNOS in SMG. **A:** a granular convoluted tubule and **B:** striated ducts (1200×).

that was of very short duration and was not significant by the next stimulation. The same pattern of effect was observed with NA (see FIGURE 4). Therefore, we can conclude that NO exerts a stimulatory action on salivary secretion in normal male rats.

Effect of LPS on Salivary Secretion

Injection of LPS (5 mg/kg i.p.) to induce endotoxemia, 6 h before the measurement of salivary secretion stimulated with MC or NA as described above for the

TABLE 1. Relative levels of NOS immunoreactivity in SMG slices

Distribution	nNOS	iNOS
Nerve fibers	++	−
Apical membrane of excretory and striated ducts	++	+++
Cytoplasm of excretory and striated ducts	+	+
Cytoplasm of granular convoluted tubules	++	++
Macrophages	−	+

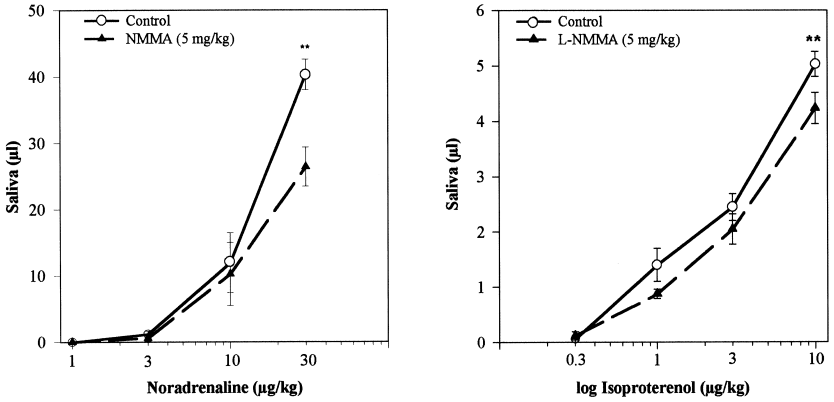

FIGURE 3. The effect of L-NMMA on salivary secretion induced by NA and isoproterenol. Values are means ± SEM of six experiments. $^{**}p < 0.01$ vs. the respective control.

nitroglycerin experiments, produced an almost total block of salivary secretion (data not shown).

Activity of NOS after L-NMMA, LPS and LPS Plus Aminoguanidine (AG)

By using the method of the conversion of [^{14}C]arginine to citrulline by NOS, we have shown previously that the activity of NOS after administration of L-NMMA

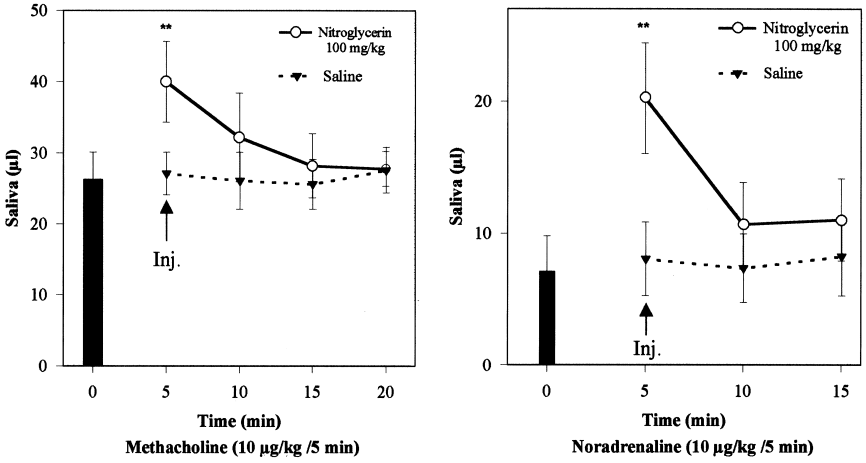

FIGURE 4. The effect of nitroglycerin on salivary secretion induced by MC or NA. Values are means ± SEM of six experiments. $^{**}p < 0.01$ vs. the respective control.

(5mg/kg) i.v. 1 h prior to sacrifice was highly significantly ($p < 0.001$) reduced.[2] Administration of LPS (5mg/kg) 6 h prior to sacrifice induced a 20% increase ($p < 0.05$) of NOS activity in the SMG. Since AG has been shown to be a specific inhibitor of iNOS *in vitro*[9] and *in vivo*,[10] we injected AG (20 mg/kg) i.p. 2 min prior to injection of LPS and measured the activity of NOS in the SMG at the same time (6 h) as for LPS alone. The administration of LPS together with AG prevented the increase of NOS as elicited with LPS alone ($p < 0.05$) as compared to the LPS group. These results suggest that the increased NOS activity after LPS could be due to the induction of iNOS in the SMG.

Effect of LPS on Immunoreactivity of iNOS in SMG

LPS was injected at the same dose and same time as for other experiments. SMGs were obtained at 6 h and immunohistochemical studies performed as described above. There was an increase in the number of macrophages stained with iNOS, but no other observable changes (see FIGURE 5).

Effect of LPS and LPS plus AG on $NO_2/N0_3$ in Plasma

Plasma levels of NO_2/NO_3 were almost undetectable in control animals. Six hours after injection of LPS there was a dramatic increase in plasma NO_2/NO_3 concentration ($p < 0.001$). When AG was administrated just prior to LPS the increase in

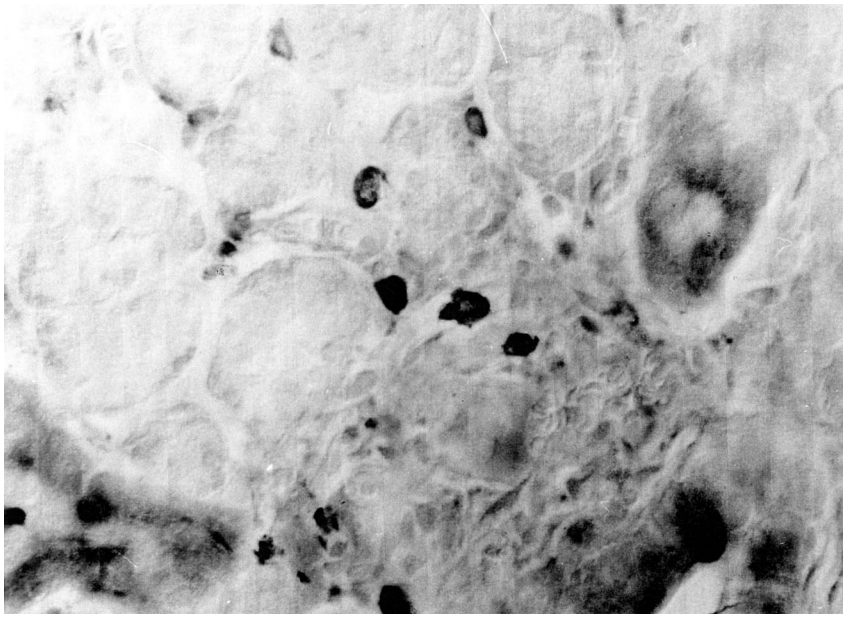

FIGURE 5. Immunohistochemical localization of iNOS in SMG showing macrophage-like cells after LPS injection. (400×)

plasma NO_2/NO_3 was significantly lower ($p < 0.001$), but still highly significantly greater than in control rats ($p < 0.001$).

Effect of LPS and LPS plus AG on PGE Content in SMG

Injection of LPS also dramatically increased PGE content at 6 h by 100% ($p < 0.001$). The injection of AG just prior to LPS blocked completely the effect of LPS as PGE content of control was identical to that of the LPS + AG group.

Effect of LPS and LPS plus AG on COX as Measured by Radioconversion of [^{14}C]-AA to Prostanoids by SMG

The radioconversion assay of [^{14}C]-AA that indirectly measures COX activity by measuring arachidonate metabolites such as 6-keto $PGF_1\alpha$, $PGF_2\alpha$ and PGE_2 gave similar results to those observed when PGE content of the SMG was measured by RIA. The results were expressed as percentage of total cpm of a particular prostanoid on plate/100 mg wet weight of the SMG. The PGE_2 values were (mean ± SEM): Control group = 7.3 ± 1, LPS = 12.2 ± 1, and LPS ± AG = 8.11 ± 0.8. The increase induced by LPS was significant ($p < 0.05$) versus the other two groups. Similar results were obtained for 6-keto-$PGF_2\alpha$ and $PGF_1\alpha$ .The increased COX activity after LPS probably represents COX2 induction by LPS, and its blockade by the inhibitor of iNOS, AG, suggests that it was induced by NO.

Effect of a PGE_1 Analogue on Salivary Secretion

Since we have found that NO has a stimulatory effect on salivary secretion and that the administration of LPS that increases NOS activity with higher production of NO paradoxically produced an almost total abolition of stimulated salivary secretion, we hypothesized that this inhibition could be caused by an inhibitory effect of PGs. Therefore, we stimulated salivary secretion by NA as previously described, and injected the PGE_1 analogue (Alprostadil, 3 μg/kg). The injection of the analogue inhibited dramatically ($p < 0.01$) the stimulated salivary secretion.

DISCUSSION

The results of our previously reported research[2] and that reported here establish the control of salivary secretion by NO. Inhibition of NOS by L-NAME or L-NMMA inhibits the secretion induced by muscarinic cholinergic agonists, SP, noradrenaline and the β-adrenergic agonist, isoproterenol. Conversely, nitroglycerin, a releaser of NO, increased stimulated salivation. NO has a relatively small role, at least in the anesthetized rat, since the effect of nitroglycerin is relatively small and NOS inhibitors only partially reduced the effects of secretogogues. There was no NOS in the acini that form the original secretion of the gland. Since NO would have to diffuse some distance to reach these cells, we hypothesize that this initial secretion is unaffected by NO. iNOS was frequently seen in macrophages within the gland. The content of this "constitutive" iNOS in the salivary gland was much higher than that seen in other organs, such as the pituitary gland,[11] perhaps because of constant exposure

of the gland to pathogens entering the oral cavity that would induce iNOS by releasing LPS or other bacterial and viral toxins.

Since saliva contains many compounds important to combat invading organisms, it was important to study the effect of LPS on salivary secretion. The same dose of LPS shown previously to produce a massive increase in iNOS mRNA in the anterior pituitary and pineal glands and to increase iNOS content,[11] increased the number of iNOS-containing macrophages in the SMG and increased the total NOS activity. This induction of iNOS by LPS was accompanied by a complete block of salivation in response to potent secretagogues.

In our previous studies, it was demonstrated that NO not only activates guanylyl cyclase leading to cGMP production that modulates secretion in glands, for example stimulating the release of FSH and LH[12] and inhibiting release of prolactin[13] from the anterior pituitary. It is probable that cGMP also mediates the stimulation of salivation evoked by NO. NO also activates another Fe^{++}-containing enzyme, cyclooxygenase, leading to production of PGs.[14] LPS induces iNOS in the SMG that directly induces NO release that should activate COX. Indeed, PGE content in the gland was elevated and we showed here that there was a markedly increased conversion of labeled arachidonate to PGs in the SMG of LPS-injected rats at the time that iNOS was increased. Furthermore, the PGE_1 analogue tested inhibited salivation produced by NA, confirming previous results.[15]

Therefore, our results are consistent with the following chain of events: LPS induces iNOS, which induces COX2, causing PG release that inhibits salivation.

If this chain of events is correct it should be possible to reduce the inhibitory effect of LPS on salivation with a specific inhibitor of iNOS or COX. Indeed, AG, a highly selective inhibitor of iNOS, decreased NOS in the gland and reduced the increase in plasma NO_2/NO_3 induced by LPS. If a dose of AG can be found to increase salivation during infection, it might be therapeutically useful by decreasing the hyposialosis induced by infection. Saliva has many useful effects in counteracting infection. For example, the NO secreted has antibacterial action in the mouth but is rapidly reduced to NO_2/NO_3, which then arrives at the acid environment of the stomach that rapidly converts NO_3 back to NO, generating antibacterial action there.[16] In addition a number of other antibacterial compounds are present in saliva, aiding in its antibacterial action.

ACKNOWLEDGMENTS

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