Effects of N ω - Nitro-L-arginine methyl ester hydrochloride on nitric oxide synthase expression in rabbit sublingual glands

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Abstract

Aim: In the present study, we investigated the expression of nitric oxide synthase in the rabbit sublingual glands and evaluated the effects of N ω - Nitro-L-arginine methyl ester hydrochloride (L-NAME) on NOS expression.

Methodology: In order to identify the morphological characteristics of sublingual glands, histological analysis of HE staining was performed. Immunohistochemistry using monoclonal mouse anti-endothelial NOS and anti-neuronal NOS was performed one day and three days after L-NAME treatment in the salivary glands of rabbits.

Results: Endothelial NOS (eNOS) immunoreactivity was observed in the secretory duct system of sublingual gland of rabbit. L-NAME administration significantly decreased NOS-positive immunoreactivities, but restored three days after L-NAME administration. Neuronal NOS (nNOS)-positive immunoreactivity was detected in the nerve fibers, however, unlike eNOS expression, L-NAME administration did not significantly decrease nNOS immunoreactivity one day after treatment.

Interpretation: The present study suggests that endogenous NOS is certainly present in sublingual glands of rabbits, and that the presence of eNOS and nNOS in the sublingual glands may be closely related with the actions of NO on regulating salivary secretion.

Key words : Immunoreactivity, N ω - Nitro-L-arginine methyl ester hydrochloride, Nitric oxide synthase, Sublingual gland

Male New Zealand
white rabbits

Treatment of
L-NAME
(NOS inhibitor)

Histological
analysis

Evaluate NOS
expression

Sublingual glands

New structural change,
but decreased NOS expression

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Introduction

Humans have three types of major salivary glands: parotid, mandibular and sublingual glands. Saliva, secreted by the salivary glands, not only contributes to digestion but also protects teeth and mucosa from exogenous bacteria and various toxicants (Mayhall, 1975; Tabak *et al.*, 1982; Wolff *et al.*, 1990). Without saliva, oral and mucosal infections may induce systemic diseases and microorganism infections may easily prevail (Hall 1993). Salivary glands also have endocrine functions by containing and secreting vascular endothelial growth factor, a multifunctional cytokine, through their own duct system or blood vessels (Taichman *et al.*, 1998). There are two types of ducts, intra- and interlobular ducts, in mammal salivary glands. Interlobular ducts consist of intercalated and striated ducts, and these ducts have the functions of exocrine and active transport (Humphrey and Williamson, 2001; Proctor and Carpenter, 2007). With these structures, the components of saliva secretion are controlled depending on the physiological or pathological situations.

Nitric oxide (NO) is a very important factor for regulating blood supply to salivary glands. NO does not directly control the secretion of saliva, but it triggers the release of parasympathetic neurotransmitters, such as acetylcholine and vasoactive intestinal polypeptide, which consequently induce atropine-resistant vasodilation and saliva secretion (Edwards and Garrett, 1993; Edwards *et al.*, 1996). NO is produced by the reaction of nitric oxide synthase (NOS) with L-arginine, and modulates various physiological functions, including neurotransmission and smooth muscle relaxation (Brennan *et al.*, 2003; Bult *et al.*, 1990; Ekblad *et al.*, 1994). Therefore, it is essential to know where NOS is synthesized and distributed in order to understand its exact function and to find therapeutic methods for xerostomia caused by various diseases (Fox *et al.*, 1984; Wijers *et al.*, 2002).

NOS has three isoforms in mammals, and endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutive forms along with the calcium-calmodulin-dependent enzymes (Förstermann *et al.*, 1991). The inducible isoform (iNOS) is closely concerned with immune response and induces NO production through defense mechanism (Knowles and Moncada, 1994). NOS is expressed in the sympathetic and parasympathetic ganglions, which control saliva secretion of sublingual glands (Alm *et al.*, 1995; Zhu *et al.*, 1996). These reports suggest that NO is directly synthesized and secreted from neurons which are innervated into sublingual glands and may be closely related with the control of blood supply and saliva secretion. However, there have been few studies on the expression of NOSs in the substructures of rabbit sublingual glands and their expressional changes after treatment with their inhibitors.

In view of the above, in this study, we investigated the expression of eNOS and nNOS in sublingual glands of rabbits. In

addition, we confirmed the expressional changes of NOS after treatment of N ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), known as a NOS inhibitor.

Materials and Methods

Experimental animals: Male New Zealand white rabbits, approximately nine in number and weighing about 3 kg were purchased from OrientBio (Seongnam, South Korea) and housed in a conventional state under standard conditions (light/dark cycle 12:12, and 22 ± 2 °C, $55 \pm 5\%$). The rabbits were cared in accordance with current international policies (NIH publication no. 85-23, 1985, revised 1996), and all procedures were conducted under approval from the Laboratory Animal Care of Soonchunhyang University.

Experimental groups and drug treatment: The animals were divided into three groups: control group, L-NAME (50 mg)-treated group sacrificed one day after treatment (L-NAME-1d), and L-NAME-treated group sacrificed three days after treatment (L-NAME-3d). L-NAME saturated in saline (50mg10ml⁻¹) was administered intraperitoneally. Issues were embedded in paraffin and sectioned using microtome (Leica Microsystems GmbH, Wetzlar, Germany) into 5-μm sections. The sections were then stained with hematoxylin and eosin according to the standard protocol.

Immunohistochemistry for nitric oxide synthase

expression: Sections from all groups were processed under similar conditions in order to ensure that immunohistochemical data remained comparable between the groups. The sections were deparaffinized and hydrated, then treated with 3 % H₂O₂ in 60 % CH₃OH for 1 hr in order to remove the activity of endogenous peroxidase. After PBS washing, the sections were incubated in 10% normal ovine serum in PBS for 1 hr. The sections were then incubated with primary antibodies in PBS with 0.3 % bovine serum albumin overnight at room temperature: mouse anti-endothelial nitric oxide synthase (eNOS, diluted 1:200, Transduction Laboratories; Lexington, KY) and mouse anti-neuronal nitric oxide synthase (nNOS, diluted 1:200, Transduction Laboratories). The sections were washed three times with PBS, then sequentially incubated with goat anti-mouse IgG (diluted 1:200, Vector Laboratories, Burlingame, CA) and streptavidin peroxidase complex (Vector Laboratories). The sections were visualized with 3, 3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) in 0.1 M Tris-HCl buffer. Digital images were captured using a BX51 light microscope (Olympus Corporation, Tokyo, Japan) equipped with a digital camera (DP72; Olympus Corporation) connected to a computer monitor.

Statistical analysis: The data were expressed as mean $\pm$ S.D. Difference between the means were analyzed by one-way analysis of variance, followed by Bonferroni's *post hoc* test. Analysis was performed using GraphPad Prism software.

(GraphPad Software, Inc., La Jolla) and significant differences were indicated at $p \leq 0.05$.

Results and Discussion

Effects of L-NAME on histological morphology in rabbit sublingual glands: Hematoxylin and eosin staining was performed in order to examine the normal histological morphology and evaluate the effects of L-NAME on morphological changes in sublingual glands of rabbits. The sublingual gland is a tubuloalveolar gland with well-developed capsule and granular duct, intercalated ducts, striated ducts, intralobular ducts were detected within the lobules. Most glandular cells consisted mucinous acinar cells, and a few serous cells were also observed (Fig. 1A). L-NAME has been commonly used to establish the hypertension animal model in various studies. Hypertension induced by L-NAME treatment may induce cellular damages or histological changes (Paulis et al., 2010; White et al., 2014). In the present study, no distinct structural changes were detected either in the L-NAME-1d nor in L-NAME-3d group compared to the control group (Fig. 1B and C).

Endothelial nitric oxide synthase (eNOS): One or three days after L-NAME administration, immunohistochemical analysis for eNOS and nNOS was performed to evaluate the expressional

changes in sublingual glands. In the control group, immunoreactivity for eNOS was clearly detected in intercalated ducts, striated ducts and intralobular ducts. In the blood vessels of intra- and inter-lobular connective tissues, eNOS immunoreactivity was also observed, but its expression was rarely observed in acinar cells (Fig. 2A). In the L-NAME-1d group, eNOS expression was detected in the same regions as in the control group, but the immunoreactivity of eNOS was significantly decreased (Fig. 2B). In the L-NAME-3d group, eNOS immunoreactivity was restored and clearly detected in the intra- and inter-lobular ducts (Fig. 2C). It has been reported that NOS was not detected in the vascular endothelial cells or secretory ducts in the rat salivary glands using NADPH diaphorase staining (Alm et al., 1995). In the present study, however, immunoreactivity of eNOS was clearly detected in the intra- and interlobular ducts as well as in the vascular endothelial cell, but not in the acinar cells of rabbit sublingual glands.

The expression of eNOS in the vascular endothelial cells of salivary glands supports the idea that endogenous NOS is closely concerned with regulating blood supply to the salivary glands (Leirós et al., 2000; Murthy et al., 1993). In addition, it has been reported that vasodilation occurs by vasoactive intestinal peptide (VIP), acetylcholine and bradykinin through the action of

Fig. 1: Structure of sublingual gland of a normal male rabbit. Hematoxylin and eosin staining in sublingual glands of control (A); L-NAME-1d (B) and L-NAME-3d (C) groups. gd-granular duct; ic-intercalated ducts; s-striated duct; iD-intralobular duct. Scale bar = 30 μ m.

NO (Rosignoli *et al.*, 2001), and that expression eNOS in the vascular endothelial cells supports it. In the present study, eNOS-positive immunoreactivity was not observed in the acinar cells, but it has been reported that endogenous NO regulates calcium influx into acinar cells of mice submandibular glands (Kim, 1995). This result suggests that endogenous NO from endothelial cells or secretory ducts may act on acinar cells in the sublingual glands.

Neuronal nitric oxide synthase (nNOS): nNOS immunoreactivity was observed only in nerve fibers and ganglion cells in the interlobular connective tissue in the control group (Fig. 3A). Unlike the result of immunohistochemistry for eNOS, nNOS immunoreactivity was not detected in any secretory ducts and vascular endothelial cells. Treatment of L-NAME did not induce significant changes of nNOS expression in either L-NAME-1d or L-NAME-3d group (Fig. 3B and 3C). L-NAME is known to be a non-selective NOS inhibitor, and it has been reported that treatment of L-NAME reduces NOS activity in the rat brain (Hsieh *et al.*, 2005). In the present study, L-NAME treatment decreased eNOS immunoreactivity, however, nNOS immunoreactivity was not markedly changed one day after L-NAME treatment. This finding is

in contrast to the general action of L-NAME, however, there were differences of sensitivity to L-NAME between the sub-regions of rat brain (Ayers *et al.*, 1997). It was reported that the effect of L-NAME is dose-dependent, and NOS activities reach a normal level 48 hr after administration of 5mg L-NAME in the rat brain (Ayers *et al.*, 1997). Based on these reasons, few changes of nNOS immunoreactivity in the L-NAME-1d group may be due to difference in sensitivity, time, or dose of L-NAME. nNOS not only catalyzes NO production in both central and peripheral nervous tissues (Förstermann, 2011), but generated NO also has multifarious physiological roles, such as LTP formation in the brain, relaxing the smooth muscles and vasodilation through peripheral nerves (Huang, 1997; Kavdia and Popel, 2004, Qu *et al.*, 2016). In addition, NO-mediated signaling regulates transcription and post-translational modification, however, uncontrolled excessive modification can induce various neurodegenerative disease (Bradley and Steinert, 2016; Nakamura and Lipton, 2008). In the present study, we did not observe nNOS expression in the intralobular structures and blood vessels, but we did confirm its expression in peripheral nerve fibers, and that it may contribute to regulate blood supply and saliva secretion through nitrgergic nerves (Förstermann *et al.*, 1994).

Fig. 2: Immunohistochemistry for eNOS in sublingual glands of control (A); L-NAME-1d (B) and L-NAME-3d (C) groups. (D) Relative optical density of eNOS-positive immunoreactivity compared to L-NAME-1d group; * indicates a significant difference versus L-NAME-1d group ($p < 0.05$). a-artery; v-vein; gd-granular duct; ic-intercalated ducts; s-striated duct; id-intralobular duct; ROD-relative optical density. Scale bar = 30 μ m.

Fig. 3: Structure of sublingual gland of a normal male rabbit. Hematoxylin and eosin staining in sublingual glands of control (A); L-NAME-1d (B) and L-NAME-3d (C) groups. gd-granular duct; ic-intercalated ducts; s-striated duct; id-intralobular duct. Scale bar = 30 μ m.

In the present study, we confirmed eNOS and nNOS expression in the sub-regions of sublingual glands. It has been reported that NO acts as a potential cytotoxic agent, however, it is also recognized as a regulator in secretion of glands and a messenger molecule in various physiological functions (Hibbs Jr *et al.*, 1988, Kitay *et al.*, 2017; Qu *et al.*, 2016). NOS expression in sublingual glands supports the idea that secretion induced by endogenous NO contributes not only to systemic endocrine secretion, but also to local exocrine secretion.

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References

- Alm, P., B. Uvelius, J. Ekström, B. Holmqvist, B. Larsson and K.E. Andersson: Nitric oxide synthase-containing neurons in rat parasympathetic, sympathetic and sensory ganglia: A comparative study. *Histochem. J.*, **27**, 819-831 (1995).
- Ayers, N.A., L. Kapás and J.M. Krueger: The inhibitory effects of N ω -nitro-L-arginine methyl ester on nitric oxide synthase activity vary among brain regions *in vivo* but not *in vitro*. *Neurochem. Res.*, **22**, 81-86 (1997).
- Bradley, S.A. and J.R. Steinert: Nitric oxide-mediated posttranslational modifications: Impacts at the synapse. *Oxid. Med. Cell. Longev.*, **2016**, 1-9 (2016).
- Brennan, P., G. Thomas and J. Langdon: The role of nitric oxide in oral diseases. *Arch. Oral Biol.*, **48**, 93-100 (2003).
- Bult, H., G. Boeckxstaens, P. Pelckmans, F. Jordaens, Y. Van Maercke and A. Herman: Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature*, **345**, 346-347 (1990).
- Edwards, A. and J. Garrett: Nitric oxide-related vasodilator responses to parasympathetic stimulation of the submandibular gland in the cat. *J. Physiol.*, **464**, 379-392 (1993).
- Edwards, A.V., G. Tobin, J. Ekstrom and S.R. Bloom: Nitric oxide and release of the peptide VIP from parasympathetic terminals in the submandibular gland of the anaesthetized cat. *Exp. Physiol.*, **81**, 349-359 (1996).
- Ekblad, E., P. Alm and F. Sundler: Distribution, origin and projections of nitric oxide synthase-containing neurons in gut and pancreas. *Neurosci.*, **63**, 233-248 (1994).
- Förstermann, U., E.I. Closs, J.S. Pollock, M. Nakane, P. Schwarz, I. Gath and H. Kleinert: Nitric oxide synthase isozymes. Characterization, purification, molecular cloning and functions. *Hypertension*, **23**, 1121-1131 (1994).
- Förstermann, U., J.S. Pollock, H. Schmidt, M. Heller and F. Murad: Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic

- fractions of bovine aortic endothelial cells. *Proc. Natl. Acad. Sci.*, **88**, 1788-1792 (1991).
- Förstermann, U. and W.C. Sessa: Nitric oxide synthases: Regulation and function. *Eur. Heart J.*, **33**, 829-837 (2011).
- Fox, R.I., F.V. Howell, R.C. Bone and P.E. Michelson: Primary Sjogren syndrome: Clinical and immunopathologic features. *Semin. Arthritis Rheum.*, **14**, 77-105 (1984).
- Hall, H.D.: Protective and maintenance functions of human saliva. *Quintessence Int.*, **24**, 813-816 (1993).
- Hibbs, J., J.B., R.R. Taintor, Z. Vavrin and E.M. Rachlin: Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87-94 (1988).
- Hsieh, C.L., C.L. Chen, N.Y. Tang, C.M. Chuang, C.T. Hsieh, S.Y. Chiang, J.G. Lin and S.F. Hsu: Gastrodiaelata BL mediates the suppression of nNOS and microglia activation to protect against neuronal damage in kainic acid-treated rats. *Am. J. Chin. Med.*, **33**, 599-611 (2005).
- Huang, E.P.: Synaptic plasticity: A role for nitric oxide in LTP. *Curr. Biol.*, **7**, 141-143 (1997).
- Humphrey, S.P. and R.T. Williamson: A review of saliva: Normal composition, flow and function. *J. Prosthet. Dent.*, **85**, 162-169 (2001).
- Kavdia, M. and A.S. Popel: Contribution of nNOS-and eNOS-derived NO to microvascular smooth muscle NO exposure. *J. Appl. Physiol.*, **97**, 293-301 (2004).
- Kim, W.J.: Regulation of calcium flux by membrane potential, cell volume and endogenous nitric oxide in submandibular acinar cells. *Int. J. Oral Biol.*, **19**, 1-6 (1995).
- Kitay, A.M., A. Link and J.P. Geibel: Activation of secretagogue independent gastric acid secretion via endothelial nitric oxide synthase stimulation in rats. *Cell. Physiol. Biochem.*, **44**, 1606-1615 (2017).
- Knowles, R.G. and S. Moncada: Nitric oxide synthases in mammals. *Biochem. J.*, **298**, 249-258 (1994).
- Leirós, C.P., F. Rosignoli, A.M. Genaro and M.E. Sales: Differential activation of nitric oxide synthase through muscarinic acetylcholine receptors in rat salivary glands. *J. Auton. Nerv. Syst.*, **79**, 99-107 (2000).
- Mayhall, C.W.: The physiological roles of saliva. *Ala. J. Med. Sci.*, **12**, 45-63, (1975).
- Murthy, K., K. Zhang, J. Jin, J. Grider and G. Makhlof: VIP-mediated G protein-coupled Ca_2^+ influx activates a constitutive NOS in dispersed gastric muscle cells. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **265**, 660-671 (1993).
- Nakamura, T. and S.A. Lipton: Emerging roles of S-nitrosylation in protein misfolding and neurodegenerative diseases. *Antioxid. Redox Signal.*, **10**, 87-101 (2008).
- Paulis, L., O. Pechanova, J. Zicha, A. Barta, R. Gardlik, P. Celec, J. Kunes and F. Simko: Melatonin interactions with blood pressure and vascular function during L-NAME-induced hypertension. *J. Pineal Res.*, **48**, 102-108 (2010).
- Proctor, G.B. and G.H. Carpenter: Regulation of salivary gland function by autonomic nerves. *Auton. Neurosci.*, **133**, 3-18 (2007).
- Qu, X.M., Z.F. Wu, B.X. Pang, L.Y. Jin, L.Z. Qin and S.L. Wang: From nitrate to nitric oxide: The role of salivary glands and oral bacteria. *J. Dent. Res.*, **95**, 1452-1456 (2016).
- Rosignoli, F., N.B. Goren and C.P. Leirós: Alterations in nitric oxide synthase activity and expression in submandibular glands of NOD mice. *Clin. Immunol.*, **101**, 86-93 (2001).
- Tabak, L.A., M.J. Levine, I.D. Mandel and S.A. Ellison: Role of salivary mucins in the protection of the oral cavity. *J. Oral Pathol.*, **11**, 1-17 (1982).
- Taichman, N.S., A.T. Cruchley, L.M. Fletcher, E.P. Hagi-Pavli, E.M. Paleolog, W.R. Abrams, V. Booth, R.M. Edwards and D. Malamud: Vascular endothelial growth factor in normal human salivary glands and saliva: A possible role in the maintenance of mucosal homeostasis. *Lab. Invest.*, **78**, 869-875 (1998).
- White, K., Y. Dempsey, P. Caruso, E. Wallace, R.A. McDonald, H. Stevens, M.E. Hatley, E. Van Rooij, N.W. Morrell, M.R. MacLean and A.H. Baker: Endothelial apoptosis in pulmonary hypertension is controlled by a microRNA/programmed cell death 4/caspase-3 axis. *Hypertension*, **64**, 185-194 (2014).
- Wijers, O.B., P.C. Levendag, M.M. Braaksma, M. Boonzaaijer, L.L. Visch and P.I. Schmitz: Patients with head and neck cancer cured by radiation therapy: A survey of the dry mouth syndrome in long-term survivors. *Head Neck*, **24**, 737-747 (2002).
- Wolff, A., P.C. Fox, J.A. Ship, J.C. Atkinson, A.A. Macynski and B.J. Baum: Oral mucosal status and major salivary gland function. *Oral Surg. Oral Med. Oral Pathol.*, **70**, 49-54, (1990).
- Zhu, B.S., W.P. Gai, Y.H. Yu, I.L. Gibbins and W.W. Blessing: Preganglionic parasympathetic salivatory neurons in the brainstem contain markers for nitric oxide synthesis in the rabbit. *Neurosci. Lett.*, **204**, 128-132 (1996).