Contribution of nitrergic nerve in canine gingival reactive hyperemia

Shigeru Shimada,1 Kazuo Todoki,2 Yoichi Omori,1 Toshizo Toyama,3 Masato Matsuo,4 Satoko Wada-Takahashi,1 Shun-suke Takahashi1 and Masaichi-Chang-il Lee5,*

1Department of Oral Science, 2Department of Nursing, Junior College, 3Department of Infection Control, Division of Microbiology, 4Department of Tissue-Engineering, Institute for Frontier Oral Science and 5Yokosuka-Shonan Disaster Health Emergency Research Center & ESR Laboratories, Graduate School of Dentistry, Kanagawa Dental University, 82 Inaoka-Cho, Yokosuka, Kanagawa 238-8580, Japan

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Reactive hyperemia reflects a compensatory vasodilation response of the local vasculature in ischemic tissue. The purpose of this study is to clarify the mechanism of regulation of this response in gingival circulation by using pharmacological analysis of reactive hyperemia and histochemical analysis of gingival tissue. Application of pressure to the gingiva was used to create temporary ischemia, and gingival blood flow was measured after pressure release. Reactive hyperemia increased in proportion to the duration of pressure. Systemic hemodynamics remained unaffected by the stimulus; therefore, the gingival reactive hyperemia reflected a local adjustment in circulation. Gingival reactive hyperemia was significantly suppressed by nitric oxide (NO) synthase inhibitors, especially the neural NO synthase-selective antagonist 7-nitroindazole, but not by anticholinergic drugs, β-blockers, or antihistaminergic drugs. Moreover, immunohistochemical staining for neural NO synthase and histochemical staining for NADPH diaphorase activity were both positive in the gingival perivascular region. These histochemical and pharmacological analyses show that reactive hyperemia following pressure release is mediated by NO-induced vasodilation. Furthermore, histochemical analysis strongly suggests that NO originates from nitrergic nerves. Therefore, NO may play an important role in the neural regulation of local circulation in gingival tissue ischemia.

**Key Words:** reactive hyperemia, nitrergic nerve, nitric oxide, gingiva

Local circulatory regulation plays an important role in the controlled delivery of oxygen, nutrients, and immunocytes that are essential to tissue homeostasis. Reactive hyperemia is a local compensatory response of the vasculature to ischemia. A number of mechanisms have been shown to mediate reactive hyperemia, including the effect of reduced oxygen tension on smooth muscle of resistance vessels,1–2 myogenic relaxation of vascular smooth muscle caused by decreased transmural pressure during artery occlusion, vasodilatory nerve stimulation by ischemia, and hypoxia-induced humoral release of vasodilatory metabolites such as adenosine.3–4 One of the important pathophysiolog-ical aspects of coronary circulation, myocardial reactive hyperemia following transient interruption of coronary blood flow, was reported to involve endothelium-derived nitric oxide (NO).5–7 It is well known that endothelial cells produce NO as endogenous endothelium-derived relaxing factor in response to stimuli such as shear stress.8–11 NO released by endothelial cells diffuses readily to the adjacent smooth muscle layer, resulting in activation of smooth muscle cell soluble guanylyl cyclase (cGC), production of the intracellular second messenger cGMP, activation of cGMP-dependent protein kinase, and ultimately in smooth muscle relaxation.12–15 NO synthases (NOS) are classified into inducible NOS (iNOS), induced by inflammation and stress, or constitutive NOS (cNOS). The neural NOS (nNOS) and the vascular endothelial eNOS are of the constitutive type.16,17

Using the NO-selective electrode, we previously demonstrated that NO mediates reactive hyperemia following pressure on the gingival tissue in rats18 or dogs.19 In that study, a slow rise in gingival tissue NO at the time of ischemia was followed by increased blood flow and sudden elevation of NO.19 Because the onset of reactive hyperemia is rapid, nervous regulation may be expected to contribute. The objectives of the present study are to prove by pharmacological and histochemical analyses that NO contributes to vasodilation during canine gingival reactive hyperemia, and to clarify the source of NO formation.

**Materials and Methods**

**Chemicals.** We purchased tetrazolium, N'-nitro-l-arginine-methyl-ester (L-NAME), 7-nitroindazole (7-NI), propranolol, atropine, pyrilamine, and cimetidine from Sigma-Aldrich (St. Louis, MO). 7-NI was dissolved in dimethyl sulfoxide (DMSO) and then diluted in 0.9% isotonic sodium chloride solution. All antagonists were prepared on the day of the experiment. nNOS antibody for histochemistry was purchased from Biorbyt (Cambridge, UK).

**Hemodynamic measurements.** The procedures used in this study were in accordance with the guidelines of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication NO. 85-23, revised 1985) and the protocols were approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental University, Yokosuka, Japan. Male beagles (8–10 kg) were anesthetized with 25 mg·kg−1 sodium pentobarbital i.v. and fixed in a supine position with the gingiva surrounding the base of the mandibular canine tooth exposed. A loop catheter was inserted into an external carotid artery, and was perfused with 1,000 U·kg−1 heparin to prevent coagulation. The loop catheter was equipped with a drug administration port, an electromagnetic blood flow meter probe (ME-26; Nihon Kohden, Tokyo, Japan), and blood pressure manometer (MPU-0.5; Nihon Kohden, Tokyo, Japan) for monitoring of systemic hemodynamics (Fig. 1A). Sodium pentobarbital was administered into the femoral vein as needed to maintain anesthesia. Gingival tissue blood flow (GBF) and oxygen partial pressure (PO2) at the base of the mandibular canine tooth were measured sequentially using a non-contact laser Doppler blood flowmeter (ALF21D; Advance, Tokyo, Japan) and tissue PO2

E-mail: lee@kdu.ac.jp

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monitor (POG-230; Unique Medical, Tokyo, Japan). External carotid artery blood flow (ECBF), external carotid artery pressure (ECBP), GBF and tissue PO$_2$ data were recorded to a personal computer hard disk through an A/D converter (PowerLab/4S; ADInstruments Japan). The recorded data were analyzed using data analysis software (Chart ver. 4.1; ADInstruments Japan).

**Reactive hyperemia protocol.** While monitoring systemic hemodynamics, gingival tissue was directly pressed for 30, 60, or 300 s with the blood flowmeter probe (1 mm diameter). The pressure was controlled so as to maintain a blood flow of 2 ml·min$^{-1}$ per 100 g in order to avoid tissue damage. Reactive hyperemia ensuing after release of pressure was evaluated in terms of three parameters: circulating blood volume (Mass), maximum blood flow (Peak), and peak half-time ($T_{1/2}$), as illustrated in Fig. 1C.$^{16,17}$ Maximum blood flow was automatically measured by a laser Doppler blood flowmeter. These parameter values were derived using data analysis software (Chart ver. 4.1). Gingival reactive hyperemia was compared between non-treated control animals and animals treated 30 min prior with antagonist. Antagonists were administered intra-arterially at the following concentrations: L-NAME, 20 mg·kg$^{-1}$; 7-NI, 20 mg·kg$^{-1}$; propranolol, 40 μg·kg$^{-1}$; atropine, 200 μg·kg$^{-1}$; pyrilamine, 2 mg·kg$^{-1}$; and cimetidine, 1 mg·kg$^{-1}$. These antagonist concentrations were inhibitory toward the following concentrations of the respective agonist: isoproterenol, 100 ng·kg$^{-1}$; acetylcholine, 300 ng·kg$^{-1}$; histamine, 300 ng·kg$^{-1}$; L-arginine, 60 mg·kg$^{-1}$ (data not shown).

**Histochemical analyses.** After measurement of gingival hemodynamics, gingival tissue in the measurement region was removed for immunohistochemical staining for nNOS and histochemical localization of NADPH diaphorase (NADPH-d) activity using tetrazolium according to the method of Law et al.$^{18}$

**Statistical analysis.** Experimental data are expressed as mean ± SEM, and compared using Student’s $t$ test or analysis of variance. $P$ values of less than 0.05 were considered statistically significant.

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**Results**

**Effects of ECBF and ECBP, GBF, PO$_2$, induced by reactive hyperemia.** ECBF and ECBP were unchanged by application of pressure to the gingiva whereas GBF decreased immediately, and tissue PO$_2$ decreased gradually after a delay. Tissue blood flow quickly became elevated following the release of pressure, and PO$_2$ increased gradually after a delay (Fig. 2). Comparing individual parameters of the reactive hyperemia response, we found that Mass and $T_{1/2}$ both increased with increasing duration of pressure over the 30- to 300-s range (Fig. 3).

**Effects of pharmacological or NOS inhibitors on parameters of the reactive hyperemia.** Parameters of gingival reactive hyperemia were unaffected by pretreatment with atropine, propranolol, pyrilamine, or cimetidine (Fig. 4 and 5). However, all three parameters of gingival reactive hyperemia were significantly reduced by L-NAME or 7-NI pretreatment (Fig. 6 and 7).

**Measurement for immunohistochemical evaluation of nNOS localization.** After in vivo experiments, gingival tissue was collected from the region of blood flow measurement for immunohistochemical evaluation of nNOS localization (Fig. 8). The gingival lamina propria and surrounding vascular tissue stained strongly positive for NADPH-d activity. Regions with a characteristic neuronal morphology and dark blue staining were identified as NADPH-d-positive neurons (Fig. 8A and C). Tissue sections of the same region also gave a strong positive immunohistochemical reaction indicating the presence of nNOS (Fig. 8B and D).

**Discussion**

Reactive hyperemia is the transient increase in organ blood flow that occurs following a brief period of ischemia, usually arterial occlusion. Hypoxia may lead to vasodilatory neuromodulation and release of vasodilatory metabolites that are thought to contribute...
to the mechanisms of reactive hyperemia. It is possible that reactive hyperemia is a compensatory mechanism for increasing blood flow to the ischemic tissue. The reactive hyperemia response would be blunted in patients with cardiovascular risk factors.\(^{19,20}\)

In our experimental model, pressure on gingival tissue led to an increase in GBF without any changes in ECBF or ECBP, confirming the absence of systemic hemodynamic effects. Therefore, the specific increase in GBF during gingival reactive hyperemia clearly reflected local circulatory regulation. Our pharmacological study showed that this gingival reactive hyperemia was completely unaffected by pretreatment with the muscarinic receptor blocker atropine, the anticholinergic β receptor blocking agent propranolol, the H\(_1\) receptor blocking agent pyrilamine, and the H\(_2\) receptor blocking antihistaminic agent cimetidine, indicating that gingival reactive hyperemia occurs via a nonadrenergic, noncholinergic, and nonhistaminergic mechanism. On the other hand, gingival reactive hyperemia was significantly inhibited by the non-specific NOS inhibitor L-NAME as well as the nNOS-specific inhibitor 7-NI. These results strongly suggest that a nitrergic nervous component contributes to the regulation of gingival circulation. This hypothesis is also strongly supported by the histochemical and immunohistochemical localization of both nNOS protein and NADPH-d activity in the tissue. Further, the rapidity of the vascular response indicated by our analysis of reactive hyperemia parameters is consistent with nervous mediation. Blood flow rapidly attained the same peak value regardless of the duration of pressure, possibly due to maximum vasodilation immediately after release of pressure.

Fig. 2. Representative trace of ECBP (A), ECBF (B), gingival PO\(_2\) (C), and GBF (D) during an experiment. Gray zones represent intervals (30, 60, and 300 s) of pressure application to the gingiva.

Fig. 3. (A) Dependence of gingival reactive hyperemia parameters on the duration of pressure. Values represent mean ± SEM (n = 6). *p<0.05, **p<0.01 for comparison between the indicated groups. (B) Representative traces of gingival reactive hyperemia for 30, 60, and 300 s of pressure.
the other hand, both Mass and $T_{1/2}$ increased with increasing duration of pressure.

It is not yet clear how a decrease in tissue PO$_2$ due to gingival compression would lead to release of NO by nitrergic nerve. A possible mechanism is suggested by the report of Henrich et al.,$^{[21,22]}$ showing that intracellular Ca$^{2+}$ influx triggers NO release from sensory nerve cells in rats and mice. Intracellular ATP decreases in ischemic tissue, and ATP depletion inhibits activity of the Na pump (Na$^+/K^+$-ATPase). The consequent accumulation of intracellular Na$^{+}$ may cause reversal of Na$^+/Ca^{2+}$ exchange and importation of Ca$^{2+}$ into the cell. Another possible mechanism is that reactive hyperemia is largely determined by the ATP-sensitive potassium channel, probably through the effect on membrane potential and voltage-sensitive Ca$^{2+}$ channels that has been observed in dogs$^{[23]}$ and humans.$^{[24]}$ The ATP-sensitive potassium channel is also involved in activation of the voltage-
dependent Ca\textsuperscript{2+} channel. Finally, elevated neuronal intracellular Ca\textsuperscript{2+} may activate NOS in conjunction with calmodulin, resulting in NO production and release.\textsuperscript{(25,26)}

Hypoxia-inducible factors (HIFs) are transcription factors induced during tissue ischemia.\textsuperscript{(27,28)} HIF-1 was first discovered in 1992 as a regulator of the erythropoietin gene, but HIF-1 also controls transcription of a number of other genes, including vascular endothelium growth factor (VEGF), a factor involved in angiogenesis and cell growth in normal and carcinoma tissue.\textsuperscript{(29)} In addition, HIFs regulate transcription of several vasoactive proteins such as adrenomedullin, endothelin, and NOS-related protein.\textsuperscript{(30)} Therefore, in reactive hyperemia, HIFs may contribute to control of NO release from nitrergic nerves. It may be possible to elucidate the actual role of HIF in nitrergic nerve function in gingival tissue. In this study, the application of the hypoxic condition was too short and the duration of the hypoxia was not sufficient to induce transcription and translation of NOS. Further study is needed to ascertain whether the involvement of HIFs in the regulation of NO is associated with reactive hyperemia.

In conclusion, this study demonstrates the potential for release of NO from nitrergic nerves in gingival tissue under hypoxic conditions.
of NO by nitrergic nerve in canine gingival tissue, and presents evidence for its probable participation as a primary local regulator of circulation in gingival reactive hyperemia.

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Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| cGC          | cell soluble guanylyl cyclase |
| cNOS         | constitutive nitric oxide synthase |
| ECBF         | external carotid artery blood flow |
| ECBP         | external carotid artery pressure |
| GBF          | gingival tissue blood flow |
| iNOS         | inducible nitric oxide synthase |
| L-NAME       | L-arginine methyl ester |
| Mass         | circulating blood volume |
| NADPH-d      | NADPH diaphorase |
| 7-NI          | 7-nitroindazole |
| nNOS         | neural nitric oxide synthase |
| NO           | nitric oxide |
| NOS          | nitric oxide synthase |
| Peak         | maximum blood flow |
| PO₂          | oxygen partial pressure |
| T₁/₂         | peak half-time (T₁/₂) |

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Wolin MS. Activated oxygen metabolites as regulators of vascular tone. Klin Wochenschr 1991; 69: 1046–1049.
2 Ewald U, Tuve P. Reduced vascular reactivity in diabetic children and its relation to diabetic control. Acta Paediatr Scand 1985; 74: 77–84.
3 Schaper W. Natural defense mechanisms during ischemia. Eur Heart J 1983; 4 Suppl D: 73–78.
4 Shimamoto K, Miura T, Miki T, Iimura O. Activation of kinins on myocardial ischemia. Agents Actions Suppl 1992; 38: 90–97.
5 Pagliaro P, Battuto D, Rastaldo R, Losano G. Involvement of nitric oxide in ischemic preconditioning. Ital Heart J 2001; 2: 660–668.
6 Kaski JC. Myocardial ischaemia in the hypertensive patient--the role of coronary microcirculation abnormalities. Eur Heart J 1993; 14 Suppl J: 32–37.
7 Horowitz JD, Chikov YY, Kennedy JA, Sverdlov AL. Modulation of myocardial metabolism: an emerging therapeutic principle. Curr Opin Cardiol 2010; 25: 329–334.
8 Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. J Physiol Pharmacol 2002; 53: 503–514.
9 Furchgott RF. Introduction to EDRF research. J Cardiovasc Pharmacol 1993; 22 Suppl 7: S1–S2.
10 Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. Handb Exp Pharmacol 2006; 176: 213–254.

Fig. 8. (A and C) Histochemical stain for NADPH-d activity in canine gingival tissue (×100). (B and D) Immunohistochemical stain for nNOS in the same fields (×100). Arrows indicate cells positive for nNOS and NADPH-d; arrowheads indicate nerve fibers surrounding the blood vessels.
11 Harrison DG, Widder J, Grumbach I, Chen W, Weber M, Searles C. Endothelial mechanotransduction, nitric oxide and vascular inflammation. J Intern Med 2006; 259: 351–363.
12 Feil R, Feil S, Hofmann F. A heretical view on the role of NO and cGC in vascular proliferative diseases. Trends Mol Med 2005; 11: 71–75.
13 Murad F. Nitric oxide and cyclic guanosine monophosphate signaling in the eye. Can J Ophthalmol 2008; 43: 291–294.
14 Tews DS. Role of nitric oxide and nitric oxide synthases in experimental models of denervation and reinnervation. Microsc Res Tech 2001; 55: 181–186.
15 Wang Y, Marsden PA. Nitric oxide synthases: gene structure and regulation. Adv Pharmacol 1995; 34: 71–90.
16 Sugiyama S, Takahashi S, Tokutomi F, et al. Gingival vascular functions are altered in type 2 diabetes mellitus model and/or periodontitis model. J Clin Biochem Nutr 2012; 51: 108–113.
17 Omori Y, Takahashi SS, Todoki K. Role of nitric oxide in post-ischemic gingival hyperemia in anesthetized dogs. Redox Rep 2002; 7: 300–303.
18 Law AS, Baumgardner KR, Meller ST, Gebhart GF. Localization and changes in NADPH-diaphorase reactivity and nitric oxide synthase immuno-reactivity in rat pulp following tooth preparation. J Dent Res 1999; 78: 1585–1595.
19 Huang AL, Silver AE, Shvenke E, et al. Predictive value of reactive hyperemia for cardiovascular events in patients with peripheral arterial disease undergoing vascular surgery. Arterioscler Thromb Vasc Biol 2007; 27: 2113–2119.
20 Hirota K, Kudo M, Hashimoto H, Kushikata T. A marked increase in gastric fluid volume during cardiopulmonary bypass. J Clin Biochem Nutr 2011; 49: 16–19.
21 Henrich M, Paddenberg R, Haberberger RV, et al. Hypoxic increase in nitric oxide generation of rat sensory neurons requires activation of mitochondrial complex II and voltage-gated calcium channels. Neuroscience 2004; 128: 337–345.
22 Henrich M, Hoffmann K, Konig P, et al. Sensory neurons respond to hypoxia with NO production associated with mitochondria. Mol Cell Neurosci 2002; 20: 307–322.
23 Aversano T, Ouyang P, Silverman H. Blockade of the ATP-sensitive potassium channel modulates reactive hyperemia in the canine coronary circulation. Circ Res 1991; 69: 618–622.
24 Bijlstra PJ, den Arend JA, Lutterman JA, Russel FG, Thien T, Smits P. Blockade of vascular ATP-sensitive potassium channels reduces the vaso-dilator response to ischaemia in humans. Diabetologia 1996; 39: 1562–1568.
25 Presta A, Liu J, Sessa WC, Stuehr DJ. Substrate binding and calmodulin binding to endothelial nitric oxide synthase coregulate its enzymatic activity. Nitric Oxide 1997; 1: 74–87.
26 Zubrow AB, Delivoria-Papadopoulos M, Fritz KJ, Mishra OP. Effect of neuronal nitric oxide synthase inhibition on CA2+/calmodulin kinase kinase and CA2+/calmodulin kinase IV activity during hypoxia in cortical nuclei of newborn piglets. Neuroscience 2004; 125: 937–945.
27 Semenza GL. Surviving ischemia: adaptive responses mediated by hypoxia-inducible factor 1. J Clin Invest 2000; 106: 809–812.
28 Paul SA, Simons JW, Mahjess NJ. HIF at the crossroads between ischemia and carcinogenesis. J Cell Physiol 2004; 200: 20–30.
29 Mohamed KM, Le A, Duong H, Wu Y, Zhang Q, Messadi DV. Correlation between VEGF and HIF-1alpha expression in human oral squamous cell carcinoma. Exp Mol Pathol 2004; 76: 143–152.
30 Manalo DJ, Rowan A, Lavole T, et al. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 2005; 105: 659–669.