Expression Profiles of NOS Isoforms in Dental Pulp and Odontoblasts in nNOS Knockout Mice

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Abstract

Active oxygen and free radicals are involved in metabolism in cells and tissues. Immunohistological studies of related enzymes are few, and the morphological dynamics of these enzymes in dental pulp and odontoblasts remain to be elucidated. Nitric oxide synthase (NOS) has 3 isoforms: nNOS, iNOS, and eNOS. The aim of this study was to investigate the profiles of NOS isoforms in the absence of nNOS in dental pulp and odontoblasts. Five-week-old male C57BL/6 and nNOS knockout (KO) mice were sacrificed and expression of nNOS, iNOS, and eNOS determined immunohistochemically. Expression of nNOS was positive, whereas that of iNOS was negative and eNOS weakly positive in the dental pulp and odontoblasts of the control mice. In nNOS KO mice, expression of iNOS was positive in dental pulp and strongly positive in odontoblasts, whereas that of eNOS was stronger in fibroblasts, endothelial cells in the vicinity of blood vessels in the dental pulp, and odontoblasts. Expression of nNOS was negative in the nNOS KO mice. This suggests that iNOS and eNOS compensate for nNOS deficiency in vascular endothelial cells and fibroblasts in the dental pulp and odontoblasts.

Key words: Nitric oxide synthase — Knockout mice —
Dental pulp — Odontoblasts — Immunohistochemistry

Introduction

Nitric oxide (NO), reactive oxygen, and antioxidative enzymes are induced together or inhibit each other, forming a complex network, and NO is closely involved in not only various diseases²⁾, but also a wide range of physiological functions, including neurotransmission¹⁷⁾, immune response¹¹⁾, adjustment to the vascular system⁷⁾, apoptosis³⁾, and wound healing⁹⁾.

Nitric oxide is synthesized by NO synthase (NOS) in the body, of which three distinct isoforms have been reported in mammalian tissues^{6,8)}: neuronal NOS (nNOS), which is constantly expressed in neurons; endothelial NOS (eNOS), which is expressed in vascular endothelial cells; and inductive NOS (iNOS) which is induced in inflammation reactions. These three isoforms compensate each other^{19,20)}, and their clarification has enabled elucidation of specific individual NO production systems.

Reactive oxygen and free radicals are believed to be involved in metabolism and transport in the tissues of the oral cavity, particularly in endothelial cells and fibroblasts in the dental pulp, but the dynamics of the related enzymes remain to be clarified¹⁾. Only eNOS and iNOS have been investigated in immunohistochemical studies of endothelial cells and fibroblasts in human dental pulp: the results showed that they were positive for eNOS^{4,5)}, but negative for iNOS⁴⁾. While the presence of nNOS has been suggested based on investigation of NADPH-d¹⁴⁾, this remains to be demonstrated. Although iNOS and eNOS have been shown to compensate for nNOS deficiency in the gingiva of nNOS knockout mice¹³⁾, the relationship between cells expressing nNOS, iNOS, and eNOS and their synthases in dental pulp and odontoblasts remain to be clarified.

In the present study, it was hypothesized that iNOS and eNOS compensated for nNOS deficiency in dental pulp and odontoblasts. To investigate the dynamics of the other NO synthases in the absence of nNOS, expression of nNOS, iNOS, and eNOS was immunohisto-

chemically investigated in vascular endothelial cells and fibroblasts in dental pulp and odontoblasts using nNOS knockout (KO) mice.

Materials and Methods

1. Animal treatment

All animals in these experiments were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College (No.160204).

2. Specimen preparation

The control and experimental groups comprised 6 C57BL/6 mice and 6 nNOS KO mice (Stock no.2633, Jackson Lab., Bar Harbor, USA)¹²⁾, respectively. All mice were 5-week-old males weighing approximately 20–25 g each. None of the mice in either the experimental or control group showed any evidence of inflammation, such as flare or swelling.

The animals were anesthetized with an overdose of thiopental (Somnopenethyl 64.8 mg/ml: Kyoritsu Seiyaku, Tokyo, Japan) diluted to 10% in saline and administered at 0.05 ml per 10 g weight intraperitoneally. The maxilla and molar teeth (M1) were then excised and fixed by perfusion with 4% paraformaldehyde solution. They were then cut into 3.0-mm³ pieces and fixed in 4% paraformaldehyde solution at room temperature for 2 hr. After washing, the samples were decalcified with 10% EDTA at 4°C for 2 weeks, dehydrated with alcohol by the conventional method, and then embedded in paraffin for preparation of thin sections.

3. Immunohistochemistry

Protein expression levels were investigated by immunohistochemistry. The paraffin sections were deparaffinized with xylene and ethanol and then reacted with glycine PBS for 30 min. After that, they were reacted with 0.1% NaN₃ for 30 min and washed with PBS

Fig. 1 NOS protein expression in central region of dental pulp

Positive reaction for nNOS was detected in endothelial cells (arrowhead) and fibroblasts (arrow) in vicinity of blood vessels in control group (a). Negative reaction for nNOS was observed in endothelial cells and fibroblasts in vicinity of blood vessels in nNOS KO group (b).

Negative iNOS expression was observed in control group (c). Positive reaction for iNOS was detected in endothelial cells (arrowhead) and fibroblasts (arrow) in vicinity of blood vessels in nNOS KO group (d).

As for eNOS, weakly positive reaction was noted in endothelial cells (arrowhead) and fibroblasts (arrow) in vicinity of blood vessels in control group. Strongly positive reaction was observed in endothelial cells (arrowhead) and fibroblasts (arrow) in vicinity of blood vessels in nNOS KO group.

Scale bar = 50 μ m

Immunopositive staining is indicated by a dark brown color.

buffer. After washing, they were incubated in 1% non-fat dry milk for blocking for 1 hr at room temperature. They were then washed with PBS buffer again and reacted with the primary antibody. For nNOS staining, rabbit anti-nNOS polyclonal IgG was used^{15,16)}; for iNOS, rabbit anti-iNOS polyclonal IgG was used^{18,21)}; for eNOS, mouse anti-eNOS monoclonal IgG was used^{10,12)}.

Sections were inspected using a Universal

Microscope: Axiophot 2 and AxioCam (ZEISS).

Results

A positive reaction revealing expression of nNOS was detected in endothelial cells and fibroblasts in the vicinity of blood vessels in the central region of the dental pulp in the

Fig. 2 NOS protein expression in odontoblasts

Positive reaction was observed for nNOS in control group (arrows) (a), whereas negative reaction was evident in nNOS KO group (b).

Negative expression of iNOS was observed in control group (c), whereas strongly positive expression was seen in basal region of cellular processes (arrows) in nNOS KO group (d). Weakly positive reaction was noted for eNOS in control group (e), and strongly positive reaction in basal region of cellular processes (arrow) in nNOS KO group.

Scale bar = 50 μ m

Immunopositive staining is indicated by dark brown color.

control group (Fig. 1a). Staining was completely negative for nNOS protein in the nNOS KO group (Fig. 1b).

Negative expression of iNOS was observed in the central region of the dental pulp in the control group (Fig. 1c). In the nNOS KO group, a positive reaction revealing expression of iNOS was detected in endothelial cells and fibroblasts in the vicinity of blood vessels (Fig. 1d).

As for eNOS expression in the central region of the dental pulp, a weakly positive reaction was noted in endothelial cells and fibroblasts in the control group (Fig. 1e). In

the nNOS KO group, the reaction was stronger in endothelial cells and fibroblasts (Fig. 1f) than in the control group.

A positive reaction revealing expression of nNOS was detected in odontoblasts in the control group (Fig. 2a). Staining was completely negative for nNOS protein in the nNOS KO group (Fig. 2b).

Negative iNOS expression was observed in odontoblasts in the control group (Fig. 2c). In the nNOS group, strongly positive iNOS staining was observed in the basal region of cellular processes in odontoblasts (Fig. 2d).

As for eNOS expression in odontoblasts, a

weakly positive reaction was noted in the control group (Fig. 2e). In the nNOS KO group, the reaction was stronger in the basal region of cellular processes in odontoblasts (Fig. 2f) than in the control group.

Discussion

Expression of nNOS was positive in endothelial cells and fibroblasts in dental pulp in the control group. According to Law *et al.*, NADPH-d was present in rat dental pulp, suggesting the presence of nNOS¹⁴. Based on this earlier finding, immunohistochemical staining using anti-nNOS antibodies was performed in the present study to determine the presence or absence of nNOS in dental pulp. The results revealed a positive reaction for nNOS in endothelial cells and fibroblasts. This suggests that these cells are affected by nerve fibers containing nNOS, just as fibroblasts and endothelial cells are in other tissues.

Expression of eNOS and nNOS was positive in the basal region of odontoblasts in the control group. This was likely due to the special oxygen environment caused by protrusion of odontoblasts into dentinal tubules.

In the nNOS KO mice, expression of nNOS was negative in endothelial cells and fibroblasts, which is not contradictory since nNOS was knocked out in these mice.

Negative iNOS expression was observed in endothelial cells and fibroblasts in dental pulp and odontoblasts in the control group. This is consistent with a previous immunohistochemical study which also reported negative expression of iNOS in normal dental pulp⁴. In the nNOS KO mice in the present study, the reaction was positive or strongly positive in endothelial cells, fibroblasts, and odontoblasts. This suggests that iNOS compensated for nNOS deficiency against a decrease in NO production due to the absence of nNOS. The finding that iNOS produced much more NO than the other isoforms indicates that NO production increases with a decrease in nNOS. One earlier study

found that iNOS increased in dental pulp during inflammation⁴. However, the present results showed that iNOS increased when the balance of the various isoforms of NOS was broken by nNOS KO rather than inflammation.

Expression of eNOS was weakly positive in endothelial cells, fibroblasts, and odontoblasts in the control group. Previous immunohistochemical studies such as those by Felaco, M. *et al.*³⁾ and Di Nardo *et al.*⁴⁾ reported the presence of eNOS in human dental pulp, and similar results were obtained here, also. The reaction was more strongly positive in endothelial cells, fibroblasts, and odontoblasts in the nNOS KO mice than in the control mice. This suggests that the knocking out of nNOS increased expression of eNOS to maintain a constant NO level, indicating that eNOS compensates for nNOS deficiency.

Nitric oxide, reactive oxygen, and antioxidative enzymes form a complex network in the body. This study detected protein levels by immunohistological staining of cells, but investigation of mRNA by Northern blot and *in situ* hybridization may be necessary. The relationship between the expression of NOS and NO in various oral diseases remains unclear, and the production of, and pathology associated with NO have only been partially elucidated. The oral cavity is a space in which marked changes have been detected in NO levels, and each tissue is constantly influenced by NO. Nitric oxide is closely associated with the oral cavity, and further characterizing its role in this environment will require the application of a wide range of research methods in future study.

Conclusion

The results of this study confirmed that increased expression of iNOS and eNOS compensates for nNOS deficiency in vascular endothelial cells and fibroblasts in dental pulp and odontoblasts.

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