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Nitric oxide synthase expression is increased by occlusal force in rat periodontal ligament

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Structured Abstract

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Objectives – The goal of this study was to investigate the role of nitric oxide synthase (NOS) in occlusal force-induced signal transduction in rat periodontal ligament (PDL).

Design – Rats were fitted with a bite plate and a metal cap to the maxillary and mandibular incisors, respectively, to eliminate the occlusal forces on rat molars. One group was sacrificed at 7 days (exclusion group), while the remaining rats had their appliances removed to reestablish molar occlusal contact (reload group) and were sacrificed 7 days thereafter. Another group of rats (normal group) were left completely untreated. Frozen cross sections of the upper first molars were stained with NADPH-diaphorase to quantify NOS activity. The distal sides of the disto-palatal roots of the upper first molars were examined, and the number and the area of stained cells in the PDL were measured.

Results – In the normal group, NOS expression was detected in blood vessels, monocyte-macrophages, fibroblastic cells and osteoclastic cells. NOS expression was lower in the exclusion group when compared with the normal group or the reload group ($p < 0.05$), and the exclusion group exhibited occluded blood vessels and a narrowing of PDL. In contrast, in the reload group the PDL and blood vessel structure had recovered and NOS expression was increased to the level of the controls.

Conclusion – Occlusal force resulted in increased NOS expression. NO may mediate changes in PDL structure in response to occlusal force.

Key words: exclusion of occlusal force; nitric oxide synthase; reload of occlusal force

Introduction

In cases of open bite or high canines orthodontic treatment must be aimed to establish intercuspation to yield acceptable masticatory function and esthetics. Animal studies of non-occluded teeth characteristically exhibit various changes in the periodontal ligament (PDL): narrowing of the PDL space (1), decrease in the number of blood vessels, increased vasoconstriction (2), increased endothelin expression (3), changes in the extracellular matrix (4), and deformation of PDL nerve endings (5). It has also been reported that monocyte-macrophage lineage cells may play a role in the recovery changes with the transition from the non-occluded to the occluded conditions (6).

While the mechanical stress of occlusal force has been reported to be essential for the maintenance of PDL morphology and function, the signal transduction pathways that mediate this response remain unknown. Nitric oxide (NO) is a molecule, which was originally characterized as a vasodilation factor (7) plays a significant role in various signal transduction pathways (8). For example, it has been reported that NO plays a role in mechanical signal transduction leading to cartilage and meniscus degeneration (9). In the PDL mechanical stress promotes an increase in nitric oxide synthase (NOS) expression *in vitro* (10–12), and administration of NOS inhibitors prevent tooth movement (13, 14). The relationship between occlusal force and NO, however, is not clear. The purpose of this study therefore, was to investigate the role of NOS in occlusal force-mediated changes in the PDL.

Materials and methods

All procedures followed the guidelines set forth by the Tokyo Medical and Dental University Committee for Animal Research.

Animals and appliances

Thirty-six 7-week-old Sprague-Dawley rats were randomly assigned into one of three groups: the normal group (n = 12), the exclusion group (n = 12) or the reload group (n = 12). In the latter two groups, an anterior bite plate and a metal cap composed of band material (0.180 × 0.005 in; Rocky Mountain, Morita, Tokyo, Japan) were attached to the maxillary and mandibular incisors, respectively, using light-curing composite resin (Clearfil Liner Bond II, Kuraray Co. Ltd, Japan) according to the method of Suhr *et al.* (6) (Fig. 1). These appliances served to exclude occlusal force from the molar region. In the exclusion group, rats were sacrificed after 7 days of occlusal contact exclusion. In contrast, after 7 days of occlusal contact exclusion, the reload group underwent reestablishment of occlusal force by removal of the appliances. These rats were sacrificed after 7 days of reload of occlusal force. The normal group consisted of 12 untreated rats (7–9-weeks old). All animals were fed *ad libitum* with powder diet (CE-2, Clea Japan INC, Japan) and given free access to drinking water.

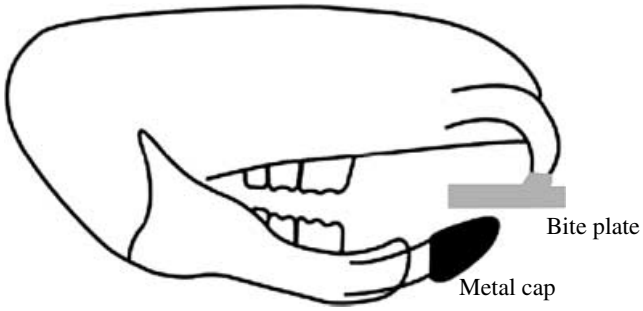

Fig. 1. An anterior bite plate on the maxillary incisor and a metal cap on the mandibular incisor produced loss of occlusal contact in the rat molar region.

Histological procedure

Rats were anesthetized and killed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The maxillae were removed, immersed *en bloc* in the same fixative solution at 4°C for 15 h and decalcified in 10% EDTA solution, pH 7.4, at 4°C for 3 weeks. The decalcified tissues were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Europe, Zeoterwoude, The Netherlands). Twenty micron frozen cross sections of the upper first molars were cut using a cryostat (CM3000, Leica, Nussloch, Germany) and mounted on poly-L-lysine coated glass slides (Matsunami, Japan). The specimens were stained with NADPH-diaphorase to detect NOS (15). The distal sides of the disto-palatal roots of the upper first molars, 500–600 μm from the furcation of the roots, were examined to evaluate the expression of

NOS. For negative control, NAD was substituted for NADP (15).

Semi-quantitative study

The enzyme-stained images were captured using a light microscope (Microphoto-FXA, Nikon, Tokyo, Japan) equipped with a digital camera (DXm1200, Nikon, Tokyo, Japan) and stored in a 24 bit true color TIFF format. Quantitative figures were obtained using image analysis software (Image-Pro, Media Cybernetics, Silver Spring, MD, USA). Two parameters were used to quantify the changes in the PDL: the number of NADPH-diaphorase positive cells and the area of NADPH-diaphorase positive cells. The parameters were measured in a pre-determined square (200 × 200 μm) that included the PDL and the alveolar bone ridge of the distal side of the disto-palatal root of the upper first molar (Fig. 2). Five representative sections obtained from each of the twelve samples of all groups were measured on three separate days.

Statistical analysis

The number and the area of NADPH-diaphorase positive cells of the three groups are shown as mean ± 1 SD. Multiple comparisons of these groups were performed using Scheffe's test ($p < 0.05$) after homoscedasticity was confirmed by analysis of variance (ANOVA; $p < 0.05$) using Stat View 5.0J software (SAS Institute, Cary, NC, USA).

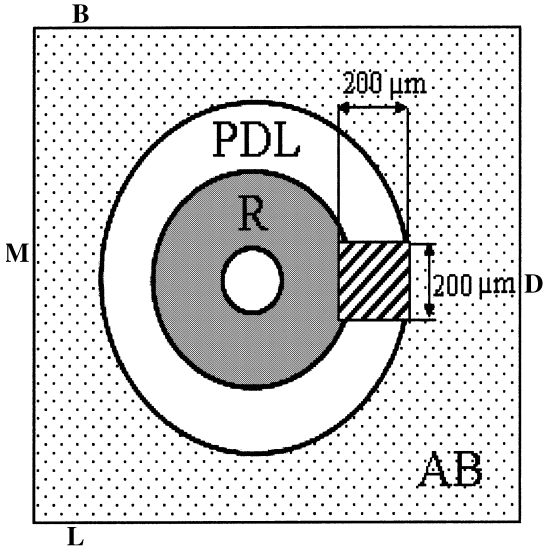

Fig. 2. A scheme of the experimental measurements. A 200 × 200 μm square (hatched area) at the distal side of the disto-palatal root of upper first molar was defined as the area for measurements. B, buccal; L, lingual; M, medial; D, distal; AB, alveolar bone; PDL, periodontal ligament; R, root.

Results

The NOS expression in the normal group PDL was detected in blood vessels, fibroblastic cells, monocyte-macrophages and osteoclastic cells (Fig. 3A arrow head). The exclusion group demonstrated narrowing of the PDL space and occlusion of blood vessels (Fig. 3B). Additionally, the number and the area of NADPH-diaphorase positive cells was significantly lower in the exclusion group (49 and 41%, respectively) when compared with the normal group ($p < 0.05$) (Fig. 4).

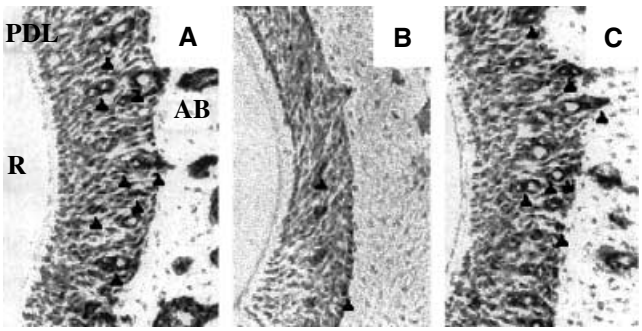

Fig. 3. Distal sides of the disto-palatal roots of the upper first molars examined with NADPH-diaphorase staining. (A) Normal group shows NOS expression in the blood vessels, monocyte-macrophages, fibroblastic cells and osteoclastic cells in the PDL. (B) Exclusion group shows blood vessels are occluded and the PDL space has narrowed. NOS expression has decreased. (C) Reload group shows strong NOS expression in the blood vessels, monocyte-macrophages, fibroblastic cells and osteoclastic cells. PDL, periodontal ligament; R, root; AB, alveolar bone; arrow head, NADPH-diaphorase positive cells.

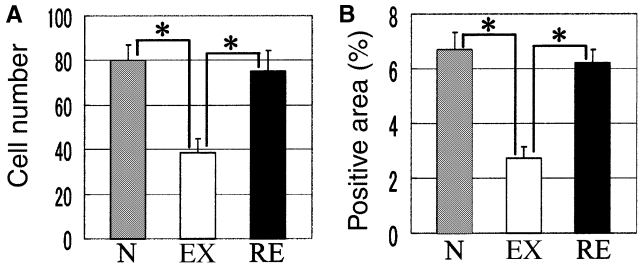

Fig. 4. (A) Number of NADPH-diaphorase positive cells (B) Area of NADPH-diaphorase positive cells (percentage of the determined area). The number and the area of NADPH-diaphorase positive cells were decreased in the exclusion group and increased in the reload group. N, normal group; EX, exclusion of occlusal force group; RE, reload of occlusal force group. Bar shows ±1 SD; *$p < 0.05$.

Animals in the reload group demonstrated recovery of the PDL space and blood vessel integrity. Further, the alveolar bone displayed a rugged profile, indicating active bone resorption (Fig. 3C). Expression of NOS in the reload group was observed in blood vessels, fibroblastic cells, monocyte-macrophages and osteoclastic cells. The number and the area of NADPH-diaphorase positive cells were significantly higher in the reload group (195 and 226%, respectively) when compared with the exclusion group ($p < 0.05$; Fig. 4). No differences were detected when comparing the normal and the reload group.

Discussion

The present study demonstrated a relationship between NOS and occlusal force as evidenced by the changes in NADPH diaphorase-positive cells with occlusal stimulation. We used measurements of enzyme activity to demonstrate the whole changes under occlusal stimulation as there are no reports on NOS expression without occlusal stimulation. This study revealed occlusal stimulation could regulate NOS expression in PDL. We observed that NOS expressed in normal rats. These data are consistent with previous studies (16, 17) and suggest that NO may play a role in occlusal force-induced maintenance of PDL integrity.

In the exclusion group, NOS was expressed less than the normal group. Loss of occlusal contact with the bite plate and the metal cap kept occlusal force away from the molar PDL. Consequently, the need for blood supply to the molar regain is decreased. Previous studies have demonstrated increased endothelin after lost occlusal contact, could act synergistically with changes in NO to produce relative vasoconstriction (3). In the reload group the PDL needs increased blood supply to recover from the state of hypofunction. This might explain the stronger expression of NOS in the exclusion group. An examination of NADPH-diaphorase positive cell data presented in Fig. 4(A, B), suggests occlusal function was the trigger in changes in cell size and proliferation.

The three different NOS isoforms, endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS) (7), each play a unique biological role in various tissues. The present study was unable to determine if selective changes in the various isoforms occur in the presence of occlusal force, as only total NOS expression was assessed. Endothelial NOS and inducible NOS, however, are candidates of the signal transduction. The morphological changes in the blood vessels of PDL were remarkable: their morphology is dictated by the PDL environment (2). Endothelial NOS has an effect in the dilation of blood vessels and is modulated by shear stress (18). An increase in the occlusal force to the PDL would increase the shear stress in the PDL microvasculature. These changes might ultimately lead to an elevation of eNOS expression in the endothelial cells. In contrast, it has been reported that iNOS is expressed in monocyte-macrophage, fibroblast and osteoclast cells (19). In this study we demonstrated that monocyte-macrophage, fibroblastic, and osteoclastic cells stain strongly. Earlier, we had reported that monocyte-macrophage lineage cells proliferate as the PDL recovers from a state of atrophy in the presence of occlusal force (6); iNOS might be a participant in this process. Contribution of the specific NOS isoforms to this phenomenon needs to be studied.

We were able to induce, with a bite plate, a hypofunctional PDL characterized by lost structure and function. Previous studies corroborate these findings (1, 2, 6), but we still need to know how a naturally hypofunctional PDL might react to being in occlusion with the opposing teeth.

In conclusion, occlusal force resulted in increased NOS expression and maintenance of PDL integrity in the rat PDL. Nitric oxide is a likely candidate to mediate occlusal force-induced changes in the rat PDL. This study supports that orthodontic treatment to bring the non-occluded teeth into intercuspation is critically important not only for masticatory function and esthetics, but also in the recovery of function and structure of the PDL.

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