The Role of Endothelial Nitric Oxide in the Substance P Induced Vasodilation in Bovine Dental Pulp

Bekir Karabucak, DMD, MS, Helmut Walsch, DDS, MS,* Yi-Tai Jou, DDS, DMD,* Shlomoh Simchon, PhD,†† and Syngcuk Kim, DDS, PhD**

Abstract

Vasodilation, an important response in neurogenic inflammation, involves release of Substance P (SP) from the sensory nerve endings. It is now well known that SP causes edema formation and vascular relaxation in nondental tissues, however, the SP vasodilatory mechanism in the dental pulp is not completely understood. Endothelium-dependent relaxation is mediated by nitric oxide (NO) release with consecutive intracellular cyclic-GMP elevation in many vascular preparations. Recently, it has been shown in different vascular systems that SP-induced vasodilation is mediated by cyclic-GMP production through different pathways involving endothelial NO or direct endothelial-independent pathways. In the present study, the role of endothelial NO in SP induced vasodilation in the dental pulp was investigated to better understand the inflammatory mechanisms. Freshly extracted bovine dental pulp was used to measure NO production. Sodium nitroprusside (SNP), L-NAME and SP were utilized to induce and to inhibit NO production in endothelial cells. Released NO byproducts were measured with chemiluminescence assay technique. The present data demonstrate that SP induces NO production by activating NOsynthase (NOS) in endothelial cells. The NOS inhibitor L-NAME blocks NO production completely. In conclusion, in the bovine dental pulp, SP-induced vascular relaxation can be mediated by inducing NOS, and subsequently NO production in endothelial cells.

Key Words

Substance P, nitric oxide, vasodilation of dental pulp, inflammation

From the *Department of Endodontics, University of Pennsylvania, School of Dental Medicine, Philadelphia, Pennsylvania; and ††Columbia University, New York.

Address requests for reprint to Dr. Bekir Karabucak, University of Pennsylvania, School of Dental Medicine, Department of Endodontics, 240 S. 40th. Street, Philadelphia, PA 19104. E-mail address: bekirk@mac.com.

Many inflammatory mediators in the dental pulp such as histamine, 5-hydroxytryptamine, prostaglandins, bradykinin, calcitonin gene related peptide (CGRP) and Substance P (SP) are released in response to pathologic, pharmacologic, and physiologic stimulation of cells and sensory nerves (1, 2). Release of these mediators leads to arteriole dilation and vascular leakage to promote healing at the injured side. However, vasodilation in the dental pulp can be harmful to pulpal tissue because of its unique "low compliance environment" (3).

SP is known to be a potent inflammatory mediator in the pulpal microvasculature and plays an important role in the pulpal inflammatory process. SP immunoreactivity was detected in the terminal sensory nerve fibers around the vessels in the central parts of the pulp and is co-localized with CGRP-like immunoreactivity (4). It was also shown that inferior alveolar nerve (IAN) stimulation increases the SP-like immunoreactivity in fluid samples collected from the feline dental pulp (1). Such SP-like immunoreactivity after IAN stimulation was confirmed when systemic use of a specific antagonist caused significant activity reduction (5). The results of recent studies also showed that SP is an important mediator. Noxious stimuli to the dental pulp can induce SP synthesis and subsequently SP release from nerve fibers (6).

Recently, researchers have suggested that SP induces vascular dilation and edema formation, because of its direct action on vascular smooth muscle cells and on endothelium-dependent relaxation factor (EDRF) (7, 8). Our previous studies on the feline pulp as well as other studies on different tissues (mesenteric vascular bed, coronary artery) indicate that SP exerts its effect through endothelial nitric oxide (NO) production (9–11). However, in human skin vascular relaxation by SP was significantly reduced after NO inhibition but not abolished, suggesting that SP mediated vasodilation might not be completely dependent on the NO pathway (12, 13).

Contrarily, some researchers reported that NO production inhibitors did not alter SP induced vasodilation in dental pulp and that there was no evidence that NO participates in vasodilation induced by tooth stimulation (14).

NO does play an important role in the maintenance of blood flow and blood pressure. An enzyme called Nitric Oxide synthase (NOS) synthesizes endogenous NO with co-substrates L-arginine, molecular O_2 and nicotine adenine dinucleotide phosphate (NADPH). L-arginine is considered a precursor in endogenous NO production, which can be synthesized within the cell with or without stimulation (15, 16). NOS has been described as membrane bound and activated by cofactor Ca^{2+}, which is available through stimulation by acetylcholine, SP or bradykinin, by shear stress and low arterial O_2 tension (17, 18). Therefore, constant and spontaneous release of NO plays an important role in the maintenance of blood flow and blood pressure. However, free radical gas has a very short half-life in physiological salt solution (19). Upon release, free NO is spontaneously converted in the presence of O_2 or O_2^- to nitrate (NO_3^-) and nitrite (NO_2^-). Nitrate and nitrite compounds (NOx) are almost inactive on platelets and vascular strips to induce vasodilation (20).

L-arginine analogues, with modification at the terminal guanidino nitrogen and/or the carboxyl terminus, have been described as inhibitors of NO in vascular tissue. They competitively block endogenous NO production by inhibiting the enzyme NOS. L-arginine analogues, especially L-NA and its methyl ester L-NAME, have been used to test NO involvement in a microvasculature and to investigate the L-arginine-NO pathway within endothelial cells (21).

Sodium nitroprusside (SNP), like other nitrovasodilators, has been used to treat cardiovascular diseases. The mechanism of SNP is not well understood, however, in biological systems, a membrane-bound enzyme with required co-factor NADH or NADPH may be involved in the generation of nitric oxide. Spontaneous generation of exogenous NO elicits vascular smooth muscle relaxation directly within the endothelial cells without involvement of the L-arginine-NO pathway (22).

SP that is released from perivascular nerve endings upon noxious stimulation, such as tooth drilling, is known to be a very potent and important neuropeptide in pulpal neurogenic inflammation. Vasodilation is a very important stage in the inflammatory process to deliver nutrients and to remove waste products from the injured side. However, the SP vasodilatory mechanism in the dental pulp is behaves differently. Our previous in vivo studies indicate that there is a correlation between SP-induced vasodilation and nitric oxide formation in the feline pulp. Furthermore, other researchers reported that SP causes vascular dilation and vascular leakage inducing cyclic-GMP production through both endothelium-dependent and independent factors in isolated vascular tissues (23, 24).

Therefore, the purpose of our study was to examine nitric oxide involvement in SP induced vasodilation in the bovine pulp to further understand cellular mechanisms of neurogenic inflammation in the dental pulp.

Materials and Methods

The experiments were conducted on bovine dental pulps. Freshly cut bovine jaws obtained from a slaughterhouse (Moyer Packing Company, Souderton, PA) were delivered on ice and immediately upon arrival the lower 8 incisor teeth were extracted using dental elevators, forceps and surgical blades. Broken and damaged teeth as well as immature roots were excluded from the study. The selected teeth were carefully cracked with a dental chisel after making grooves on the buccal and lingual surfaces using dental tungsten carbide round #2 burs with a high-speed dental handpiece (SS White Burs Inc.). Dental pulps were collected, placed into tubes and stored on dry ice to be used for the experiment within the hour. The collected pulps from one individual mandible were considered one specimen and one experimental group. There were four groups in the experiment, each containing eight incisor pulps, for a total of 32 pulps examined.

The pulps of each experimental group were dissected into small pieces with a scalpel on a sterile glass surface. The cut sections were mixed to evenly distribute the different cells and pulpal segments, i.e. coronal, apical, central and peripheral. The specimens were separated into 13 small groups of approximately equal weight and tissue volume.

Samples were weighed on an electronic scale (Mettler, Type AE 200-S, Mettler Instruments Corp. Hightstown, NJ). The weights were noted and the tissue samples with an average weight of 0.055 g $\pm$ 0.008 g, were placed into previously labeled Petri dishes. The experimental groups were substance-P (SP), SP+L-NAME, sodium nitroprusside (SNP) and control groups.

Experimental solutions were prepared freshly and stored on ice until needed for the experiments. SP, L-NAME and SNP (Sigma Chem. Co., Saint Louis, MO) were dissolved in an adequate amount of buffer until the desired concentrations were obtained. Fresh 20 mM Tris-HCL buffer solution (pH 7.4) (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.4 mg/ml soybean trypsin inhibitor (SBTI), pH 7.5, 5 mM MgCL2 - 1 mM 3-isobutyl-1-methyl xanthine, 7.5 mM phosphocreatine, 13 units/ml creatine kinase as activator, 1 mM GTP as substrate) was used to prepare the drugs and to incubate the tissues.

The Control group contained the tissue and 2 ml of the buffer solution. The value of NO production in this group was used as background. NO production was induced by adding 1 ml of SP (25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml) and SNP (25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml) to individual Petri dishes containing 1 ml of buffer and tissue. To isolate the SP effect on endothelial cells, 1 ml of L-NAME (NOS inhibitor) was mixed with the tissue and 1 ml SP. Concentrations of L-NAME (5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml) were calculated according to SP concentrations and molecular weight of the solutions.

All pulps were incubated at 37°C and 100% humidity for two hours (Blue M, Electric Company, Blue Island, IL). Test tubes were kept in the refrigerator overnight to complete the oxidation reaction. Subsequently, tubes were centrifuged at 2500 rpm (at 510G) for 10 min. One milliliter of supernatant solution was collected from each tube to measure nitrate, nitrite and all oxides formed (NOx) in a Sievers 280 NOA Nitric Oxide Analyzer (Sievers Instruments, Inc., 6185 Arapahoe Ave. Boulder, CO). The data was captured and transferred to a computer and a printer port to allow direct data printout of average values.

The differences between samples in which reactions were induced or blocked by different agents were compared. Results are presented as sample values, means and SEMs. The significance of changes was statistically evaluated by one-way ANOVA followed by Fisher's LSD comparison test for multiple comparisons ($p < 0.01$ was considered significant).

Results

The average NOx reading from the control groups ($n = 4$) was 0.46 $\pm$ 0.58 mMOL/g after incubating for 2 h. SNP induced significant NO production in the bovine dental pulp with assayed concentrations, except at the lowest concentration (25 μg) used. Even though NOx readings showed increased values (3.08 $\pm$ 0.64 μMOL/g) in the 25 μg concentration group, the generated NO was not statistically significant compared to the control group (0.46 $\pm$ 0.58 μMOL/g) ($p > 0.01$). A higher dosage of SNP resulted in increased NO released. This difference between groups was statistically significant (Table 1).

NO production was induced even by the lowest SP concentration (25 μg; 5.95 μMOL/g). This was significant when compared to the control group ($p > 0.01$). Higher SP concentrations (13.26 μMOL/g, 22.7 μMOL/g, 23.83 μMOL/g) incrementally raised the NOx production and the difference between groups was statistically significant, however, concentrations higher then 100 μg did not cause significant increases in NO production. L-NAME significantly inhibited SP induced NO production regardless of its dosage (Table 2).

TABLE 1. NOx values, mean values with standard error of the mean (SEM) obtained in the SNP group in μMOL/g

Sample	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean $\pm$ SEM
Control	1.458	0.1394	0.129	1.53	0.46 $\pm$ 0.58
SNP-25 μg	4.17	2.8	2.52	2.81	3.08 $\pm$ 0.64
SNP-50 μg	8.78	5.76	4.42	5.45	6.11 $\pm$ 1.62
SNP-100 μg	17.45	6.62	7.03	10.52	10.41 $\pm$ 4.34
SNP-200 μg	35.52	16.08	16.38	24.87	23.22 $\pm$ 7.94