

Differential cholinceptor modulation of nitric oxide isoforms in experimentally-induced inflammation of dental pulp tissue

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Abstract

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Aim The aim of the study was to investigate the role of muscarinic acetylcholine receptor (mAChR) activity in the regulation of endothelial (e), neuronal (n) and inducible (i) nitric oxide synthase (NOS) activity and expression in experimentally induced inflammation of rat dental pulp tissue.

Methodology Inflammation was induced by application of bacterial lipopolysaccharide (LPS) to the pulp. Extirpated pulp-tissue samples were incubated in saline solution until the various experiments were performed. Saline-treated pulp and healthy pulp tissues were used as controls. NOS activity was measured by the production of [U-¹⁴C]-citrulline from [U-¹⁴C]-arginine. Nitrite/nitrate assay was evaluated by the conversion of nitrate to nitrite in the presence of nicotinamide adenine dinucleotide phosphate. i-nos, e-nos and n-nos mRNA levels were measured using reverse-

transcriptase polymerase chain reaction by co-amplification of target cDNA with a single set of primers.

Results Application of LPS to the pulp increased NOS activity and nitrate production ($P < 0.001$), generated by iNOS over-activity and expression. Pilocarpine acting on mAChRs triggered a biphasic action on NOS activity and NO accumulation. At low concentrations, pilocarpine induced a negative effect associated with a decrease in i-nos mRNA level, whilst at high concentration, it produced a positive effect associated with increased e-nos and n-nos mRNA levels. In control pulp tissue, only the positive effect of pilocarpine was observed.

Conclusions Irreversible pulpitis changes mAChR conformation increasing its efficiency of coupling to transducing molecules that in turn induce activate iNOS. The capacity of pilocarpine to prevent NO accumulation and iNOS activity, by acting on mAChR mutation induced by pulpitis, might be useful therapeutically as a local treatment.

Keywords: dental pulp, (i-nos, e-nos, n-nos mRNA), mAChR, nitric oxide synthase, pilocarpine, pulpitis.

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Introduction

The nature of inflammation in the dental pulp may not be different from that in other organs. Thus, pulpitis is an adaptative immune process characterized by an increase in local blood flow (Olgart *et al.* 1991),

immunocompetent cell activation (Bergenholtz *et al.* 1991) and alteration of neuronal activity (Närhi & Hirvonen 1983).

The parasympathetic system innervates lymphoid cells and tissues, and can directly or indirectly influence one or more of these process (Sterin-Borda *et al.* 1990, Olgart *et al.* 1996). Muscarinic cholinergic activation may be involved in the composition and activation status of circulating immune cells by changing vascular tone blood flow or local release of acetylcholine (Ach) (Liu *et al.* 1990, Dantzer *et al.* 1998).

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A critical role of Ach and the vagus nerve has been demonstrated in the immune inflammatory process. Vagotomy blocks behavioral responses and cytokine induction in the brain following pro-inflammatory stimuli (Maier *et al.* 1998). There is evidence for the presence of cholinergic nerves, muscarinic receptors and Ach-degrading enzymes in pulp tissue (Photo & Antila 1972, Borda *et al.* 2007). The parasympathetic innervation in the dental pulp is distributed around small blood vessels (Luthman *et al.* 1992), and parasympathomimetic agents control the pulpal blood flow, which causes vasodilation (Edwall *et al.* 1973, Okabe *et al.* 1989, Liu *et al.* 1990). The vasodilatory action provoked by muscarinic cholinergic agonists has been shown to be dependent on the production of NO in a number of tissues (Gardiner *et al.* 1990, White *et al.* 1993). Recently, the physiological role of the parasympathetic system in healthy dental pulp has been demonstrated and results in the fact that activation of muscarinic acetylcholine receptors (mAChRs) triggers the release of pro-inflammatory mediators, including NO (Borda *et al.* 2007, Sterin-Borda *et al.* 2007). However, the role of parasympathetic innervation in pulpitis has not been defined.

Nitric oxide is known to act as an intracellular mediator that can be considered a double-edged sword: low concentrations can exert beneficial effects, but high concentrations, if they persist uncontrolled, can be detrimental, with the generation of highly toxic compounds (Colasanti & Suzuki 2000). Physiological NO is synthesized by two constitutively expressed enzymes: endothelial (eNOS) and neuronal (nNOS) NO synthetase. These isoforms of NOS can rapidly synthesize small amounts of NO for short periods of time, following receptor stimulation in healthy dental pulp (Sterin-Borda *et al.* 2007). nNOS is mainly localized in pulpal nerve fibres (Lohinai *et al.* 1997) and eNOS is detected in endothelial cells and odontoblasts of healthy dental pulp (Felaco *et al.* 2000). On the other hand, detrimental NO is produced in response to inflammatory stimuli, by inducible NOS (iNOS). iNOS produces large amounts of NO for prolonged periods (Moilanen *et al.* 1999), which can have a harmful effect on the immune response, by acting as a toxic agent during infection, with subsequent abrasion of the tooth (Carmignani *et al.* 2000, Yasuhara *et al.* 2007). These properties of iNOS make it an essential and important mediator in pulpitis, especially if it is activated at the onset of inflammation.

In this study, iNOS activity was evaluated as well as related to the intracellular accumulation of NO, and

whether the muscarinic cholinergic agonist pilocarpine effectively regulated NO synthesis, by modulating iNOS activity during pulpitis. The results may contribute in understanding modulation of the parasympathetic system in the course of chronic pulpitis, and suggest that mAChR agonists may be useful as a local treatment.

Materials and methods

Animals

Male Wistar rats from the Pharmacologic Bioterium (School of Dentistry, University of Buenos Aires, Argentina), weighing 220–260 g, were used. The animal experiments were approved by the local Animal Ethics Committee at the University of Buenos Aires. The animals were kept in the environmental conditions 23 °C/25 °C, 12 h dark/light cycle and they were provided water and food *ad libitum*. The animals were killed by cervical dislocation.

Induction of pulpitis

Under general anaesthesia induced with intramuscular (i.m.) ketamine (62.5 mg kg⁻¹) and i.m. xylocaine, the pulp of the left and right maxillary incisors was exposed using diamond burs. The entrance of the pulp chamber was enlarged, covering a length of 5 mm with K-files, up to size 40, to create sufficient space for the application of lipopolysaccharide (LPS) or saline. LPS from *Escherichia coli* O111:B4 (Sigma Chemical Co, St. Louis, MO, USA) was dissolved in sterile saline at a concentration of 10 mg mL⁻¹, and 4 µL was applied to the cavities. Sterile saline instead of LPS was applied to evaluate the effects of mechanical stimuli. Entrances to the pulp horn were sealed with temporary filling material (Cavit; ESPE, Seefeld, Germany). Animals were killed under ether anaesthesia at 6 h after pulp exposure, and the incisors were extracted. LPS application causes maximal inflammatory reaction in the coronal area of the pulp at 6 h and decreased thereafter confirming a previous report (Kawashima *et al.* 2005). Findings were characterized by disruption of odontoblasts, blood vessel dilatation and infiltration of many neutrophils. In contrast, sterile saline (used as a negative control) did not induce a severe inflammatory reaction, and only slight infiltration of neutrophils was observed in the coronal pulps at 6 h, which conformed with the previous studies (Kawanishi *et al.* 2004, Kawashima *et al.* 2005). Fresh dental pulp tissue was

kept at room temperature in Krebs Ringer bicarbonate (KRB) solution in the presence of CO₂ in oxygen, until the various experimental assays were performed. Non-treated healthy rat dental pulp tissue from the maxillary incisors (left and right), and sterile saline-treated dental pulp tissue were used as controls.

Determination of NOS activity

NOS activity was measured in rat dental pulp tissue by the production of [U-¹⁴C]-citrulline from [U-¹⁴C]-arginine, as previously described (Borda et al. 2007). Briefly, pulp was incubated for 30 min in 500 µL KRB solution that contained 18.5 kBq of L-[U-¹⁴C]-arginine. Inhibitors were added from the beginning of the incubation period, at the final concentrations indicated in the text, and the agonist pilocarpine (at different concentrations) at 10 min before the end of incubation. Incubation was carried out in a 5% CO₂ in oxygen atmosphere at 37 °C. Tissues were then homogenized in an Ultra Turrax homogenizer in 500 µm of medium that contained 20 mmol L⁻¹ HEPES, pH 7.4, 0.5 mmol L⁻¹ EGTA, 0.5 mmol L⁻¹ EDTA, 1 mmol L⁻¹ dithiothreitol, 1 mmol L⁻¹ leupeptin and 0.2 mmol L⁻¹ phenylmethylsulphonyl fluoride at 4 °C. Supernatants were applied to 2-mL columns of Dowex AG 50WX-8 (sodium form), and [¹⁴C]-citrulline was eluted with 3 mL water and quantified by liquid scintillation counting (Beckman LS 6500, New York, NY, USA).

Nitrite/nitrate analysis

After pulps were incubated in KRB (500 µL) for 30 min with or without drugs (following the protocol used for NOS activity assays), nitrite/nitrate levels were measured in tissue homogenates and in incubation medium with a commercial kit (Caiman Chemical Laboratories, Am Arbor, MI, USA). Briefly, nitrate was converted to nitrite by incubation with nitrate reductase in the presence of nicotinamide adenine dinucleotide phosphate. Lactate dehydrogenase was then used to destroy excess NADPH. Equal volumes of sample and Griess reagent were incubated at room temperature. After 10 min, absorbance was read at 550 nm. The nitrite concentration was determined by using sodium nitrate as a standard.

mRNA isolation and cDNA synthesis

Total RNA was extracted from rat dental pulp tissue by homogenization using the guanidinium isothiocyanate

method as previously described (Sterin-Borda et al. 2007): a 20-µL reaction mixture that contained 2 ng mRNA, 20 U RNase inhibitor, 1 mmol L⁻¹ dNTPs and 50 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). First-strand cDNA was synthesized at 37 °C for 60 min.

Polymerase chain reaction (PCR) procedures

NOS isoform mRNA levels were determined by a method that involved simultaneous co-amplification of both the target cDNA and a reference template (MIMIC) with a single set of primers. MIMIC for e-nos and n-nos and glyceraldehyde-3-phosphate dehydrogenase (g3pdh) was constructed using a polymerase chain reaction (PCR) MIMIC construction kit (Clontech Laboratories, Palo Alto, CA, USA). Each PCR MIMIC consisted of a heterologous DNA fragment with 5' and 3' end sequences that were recognized by a pair of gene-specific primers. The sizes of the PCR MIMIC were distinct from those of the native targets. The sequences of the oligonucleotide primer pairs used for the construction of MIMIC and amplification of NOS isoforms and g3pdh mRNA were as reported previously (Sterin-Borda et al. 2007). Aliquots were taken from pooled first-strand cDNA from the same group, and constituted one sample for PCR. A series of 10-fold dilutions of known concentrations of the MIMIC were added to PCR amplification reactions that contained the first-strand cDNA. PCR MIMIC amplification was performed in 100 µL of a solution that contained 1.5 mmol L⁻¹ MgCl₂, 0.4 µmol L⁻¹ primer, dNTPs, 2.5 U Taq DNA polymerase and 0.056 µmol L⁻¹ Taq Start antibody (Clontech Laboratories). After initial denaturation at 94 °C for 2 min, the cycle conditions were 45 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 45 s for enzymatic primer extension at 72 °C for NOS isoforms. The internal control was mRNA of the housekeeping gene g3pdh. PCR amplification was performed with initial denaturation at 94 °C for 2 min, followed by 30 cycles of amplification. Each cycle consisted of 35 s at 94 °C, 35 s at 58 °C and 45 s at 72 °C. Samples were incubated for an additional 8 min at 72 °C before completion. PCR products were subjected to electrophoresis following previously described procedures (Sterin-Borda et al. 2007). Different NOS isoform mRNA levels were normalized with the levels of g3pdh mRNA present in each sample, which served to control for variations in RNA purification and cDNA synthesis.

Drugs

Pilocarpine, atropine and aminoguanidine were purchased from Sigma; [N⁵-iminoethyl, L-ornithine] (L-NIO) and [N₂-propyl-L-arginine] (NZ) were from Tocris Cookson Inc. (Ellisville, MO, USA). Stock solutions were freshly prepared in the corresponding buffer.

Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA) and the Student–Newman–Keuls test were employed when pair-wise multiple comparison procedures were necessary. Differences between mean values were considered significant at $P < 0.05$.

Results

Figure 1 shows that NOS activity in LPS-treated pulp at 6 h was 5.8-fold higher than that of saline-treated or untreated pulp tissues. To demonstrate which isoforms of NOS might be increased in LPS-treated pulp, isolated rat dental pulp tissue was incubated with specific inhibitors of NOS isoforms. As can be seen in Fig. 1, inhibition of iNOS by aminoguanidine (1×10^{-6} mol L⁻¹) significantly decreased ($P < 0.001$) the basal activity of NOS in LPS-treated pulp, but had no effect on that from control groups. In contrast, the inhibition of eNOS by L-NIO (5×10^{-6} mol L⁻¹) significantly decreased ($P < 0.01$) the basal NOS active of both saline-treated and healthy pulp, with a small decrease in LPS-treated pulp. The inhibition of nNOS by NZ (5×10^{-6} mol L⁻¹) had no effect on NOS activity in any of the three groups.

This observation strengthened the causal relationship between the increase in iNOS activity and LPS treatment of pulp. These results encouraged confirmation of whether high NO levels were involved in the increased activity of iNOS in LPS-treated pulp. Significantly higher concentrations of total nitrite/nitrate were found in LPS-treated pulp compared with those from the control pulp (Fig. 2). Moreover, when NO levels were evaluated separately in the tissue and in the incubation medium, it was observed that 85% of NO was located in the LPS-treated pulp tissue and only 15% in the incubation medium. This indicated that NO retained a mainly cytoplasmic localization. In contrast, healthy pulp and negative control pulp showed lower

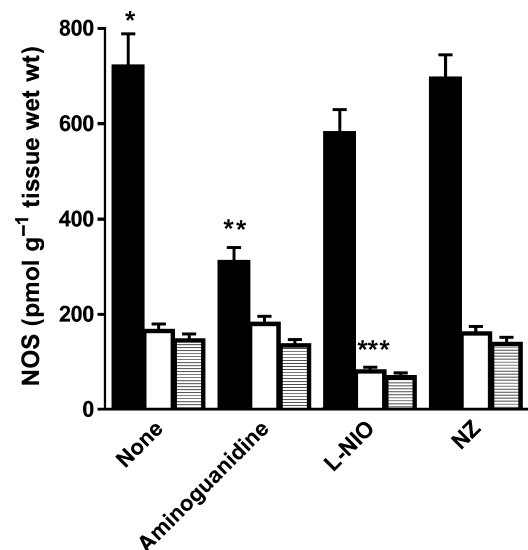


Figure 1 Basal NOS activity in LPS-treated (■), saline-treated (□) and untreated (▨) pulp, without isoform enzyme inhibitors (none) or in the presence of aminoguanidine (1×10^{-6} mol L⁻¹), L-NIO (5×10^{-6} mol L⁻¹), or NZ (5×10^{-6} mol L⁻¹). Tissues were incubated with [U-¹⁴C] arginine for 30 min with or without drugs before the assays were performed. Data shown are absolute basal values and represent the mean $\pm$ SEM of seven experiments in each group, performed in duplicate. * $P < 0.001$ versus saline-treated or untreated pulp. ** $P < 0.005$ versus LPS-treated pulp without aminoguanidine. *** $P < 0.01$ versus saline-treated pulp without L-NIO.

levels of nitrite/nitrate in tissue (17%) than in incubation medium (83%). To establish whether the elevated levels of tissue NO observed in LPS-treated pulp were generated by iNOS, the effect of different inhibitors of NOS isoforms was investigated. Figure 3 shows that the inhibition of iNOS by aminoguanidine significantly decreased ($P < 0.001$) the high concentration of nitrite/nitrate both in tissue (b) and incubation medium (c) of LPS-treated pulp, but failed to do so in saline-treated pulp. Conversely, inhibition of eNOS by L-NIO decreased the level of NO in saline-treated pulp, with slight modification of that in LPS-treated pulp. The nNOS inhibition by NZ had no effect in any of the three groups. It has been demonstrated that activation of mAChRs by pilocarpine stimulated eNOS and nNOS activity in healthy rat dental pulp. In this study, whether different NOS isoforms participated in the response of LPS-treated pulp to mAChR activation were evaluated.

Figure 4a shows the effect of increasing concentrations of pilocarpine on LPS-treated and saline-treated

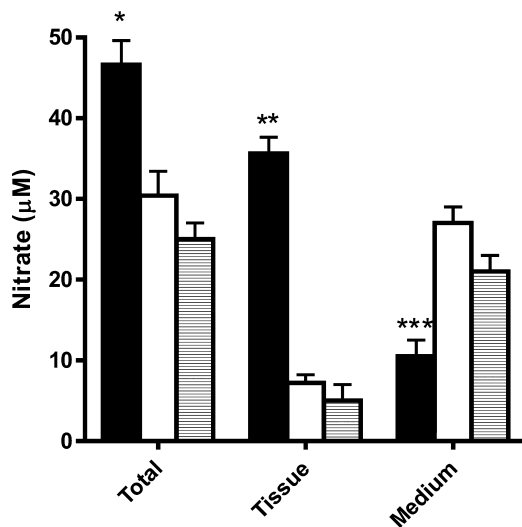


Figure 2 Basal nitrate values in LPS-treated (■), saline-treated (□) and untreated (▨) pulp in tissue incubation medium and total (sum of tissue and incubation medium values). Data shown are the absolute basal values and represent the mean $\pm$ SEM of eight experiments in each group, performed in duplicate. * $P < 0.005$ versus saline-treated or untreated pulp. ** $P < 0.001$ versus saline-treated or healthy pulp. *** $P < 0.005$ versus tissues from LPS-treated pulp.

pulp. It can be seen that in LPS-treated pulp, pilocarpine decreased NOS activity, which reached a maximal inhibition at a concentration of 5×10^{-9} mol L $^{-1}$, whilst at 1×10^{-7} mol L $^{-1}$, the mAChR agonist exerted its maximal stimulatory activity. The biphasic effect of pilocarpine was abolished by atropine (5×10^{-7} mol L $^{-1}$) (data not shown). In saline-treated pulp, pilocarpine exerted only stimulatory activity. Pilocarpine was also shown to have a biphasic effect on NOS activity in LPS-treated pulp, when nitrite/nitrate concentrations were measured. Therefore, Fig. 4b compares the negative effect of 5×10^{-9} mol L $^{-1}$ pilocarpine and its positive effect at 1×10^{-7} mol L $^{-1}$ on nitrite/nitrate concentration in LPS-treated pulp. As control, when NO levels were

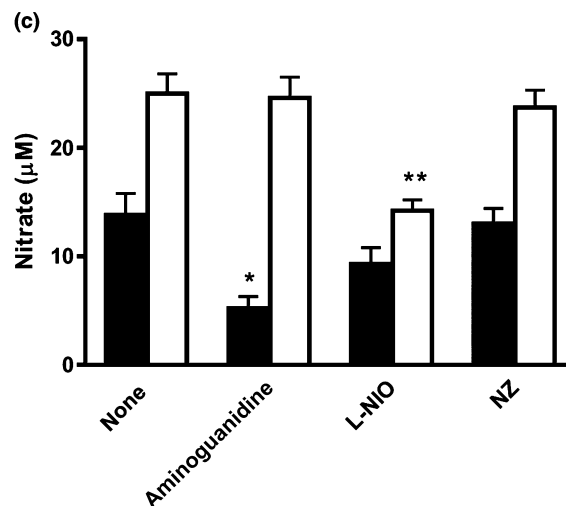
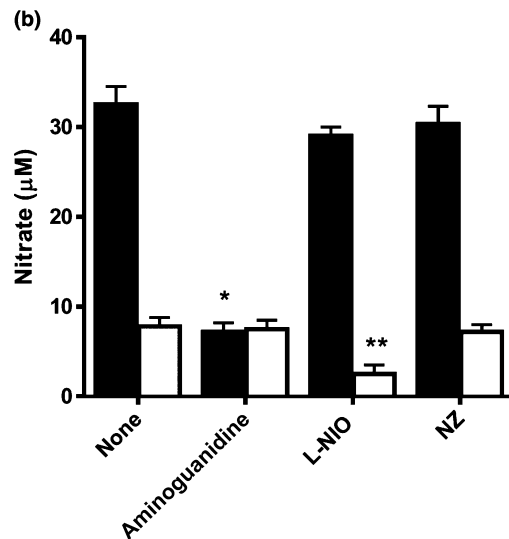
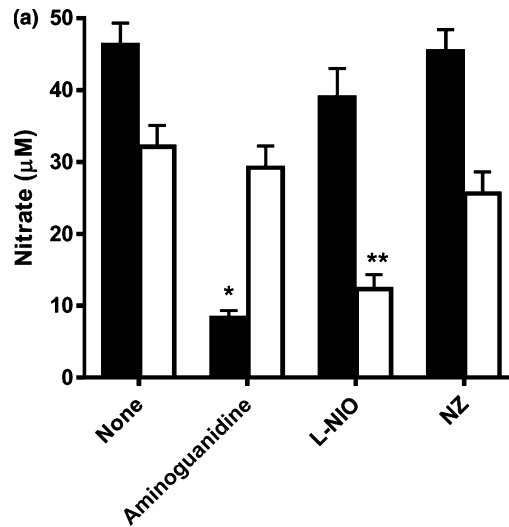


Figure 3 Effect of aminoguanidine (1×10^{-6} mol L $^{-1}$), L-NIO (5×10^{-6} mol L $^{-1}$), or NZ (5×10^{-6} mol L $^{-1}$) on basal nitrate values: (a) total, (b) tissue and (c) incubation medium from LPS-treated (■) or saline-treated (□) pulp. Data shown are the absolute basal values and represent the mean $\pm$ SEM of six experiments in each group, performed in duplicate. * $P < 0.001$ versus LPS-treated pulp without inhibitors (none). ** $P < 0.005$ versus saline-treated pulp without inhibitors (none).

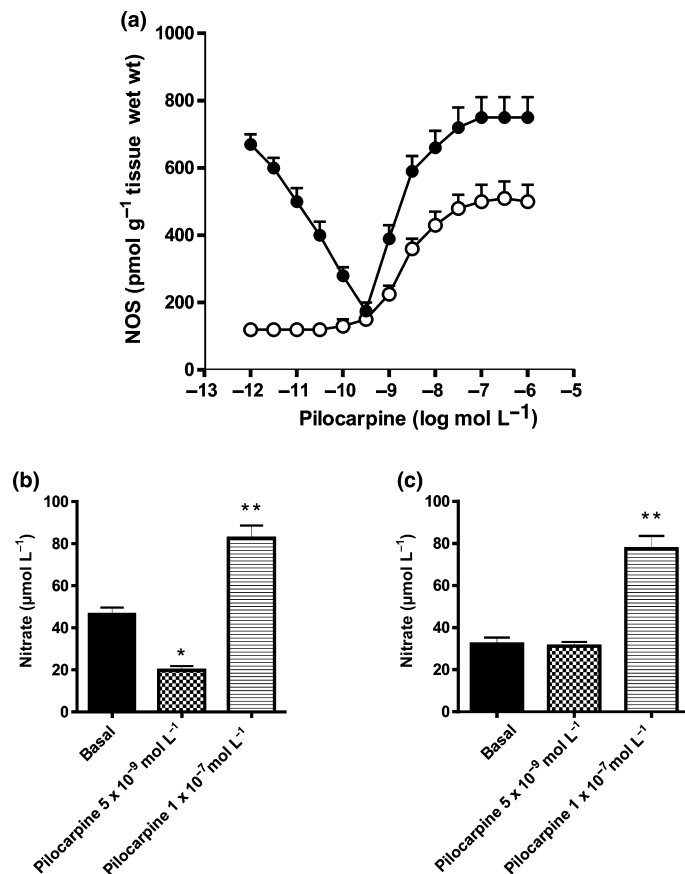


Figure 4 (a) Concentration–response curve of pilocarpine on NOS activity of LPS-treated pulp (●) compared with the response of saline-treated pulp (○). (b and c) Effects of 5×10^{-9} mol L⁻¹ pilocarpine (▨) and 1×10^{-7} mol L⁻¹ pilocarpine (▩) on nitrate concentration from LPS-treated (b) or saline-treated (c) pulp. Basal values (■) are also shown. The nitrate data are the sum of tissue and incubation medium values (total). Data shown are the mean $\pm$ SEM of six experiments in each group, performed in duplicate. * $P < 0.001$ versus basal nitrate of LPS-treated pulp. ** $P < 0.001$ versus basal values of both LPS-treated and saline-treated pulp.

evaluated on saline-treated pulp, 1×10^{-7} mol L⁻¹ pilocarpine showed positive effect, whilst 1×10^{-9} mol L⁻¹ pilocarpine had no effect (Fig. 4c).

To establish whether the increase in NO associated with the reduced response to mAChR agonist observed in LPS-treated pulp was generated by iNOS activation, the effect of 1×10^{-6} mol L⁻¹ aminoguanidine on pilocarpine-modified NOS activity was studied. The inhibition of iNOS abolished the inhibitory action of pilocarpine in LPS-treated pulp, whilst eNOS or nNOS inhibitors did not modify it (Fig. 5a). In contrast, inhibition of eNOS and nNOS significantly attenuated the stimulatory action of high pilocarpine concentrations observed in LPS- and saline-treated pulp (Fig. 5a,b).

To characterize further the role of NOS isoforms on pilocarpine action, reverse-transcriptase PCR (RT-PCR) was used on i-nos-, e-nos- and n-nos mRNA obtained from LPS- and saline-treated pulp. Using specific oligonucleotide primers, RT-PCR amplified products (Fig. 6) showed bands of the predicted size for e-nos,

n-nos and i-nos detected in dental pulp. However, whilst i-nos mRNA was detected only in LPS-treated pulp, e-nos and n-nos mRNA were detected in both LPS- and saline-treated pulp. Figure 6 also shows that in LPS-treated pulp, pilocarpine at 5×10^{-9} mol L⁻¹ decreased the size of the i-nos band, but at 1×10^{-7} mol L⁻¹, it was able to augment the size of both the e-nos and n-nos bands. On the contrary, in saline-treated pulp, pilocarpine at 5×10^{-9} mol L⁻¹ was without effect, and only augmented the size of both e-nos and n-nos bands at 1×10^{-7} mol L⁻¹, without any effect on i-nos mRNA levels.

Discussion

In spite of the relatively high density of innervation of dental pulp compared with other tissues in the body (Iijman & Zhang 2002) the role of the parasympathetic system in modulating inflamed pulp remains unknown. In this study, pharmacological evidence for the mechanism by which muscarinic

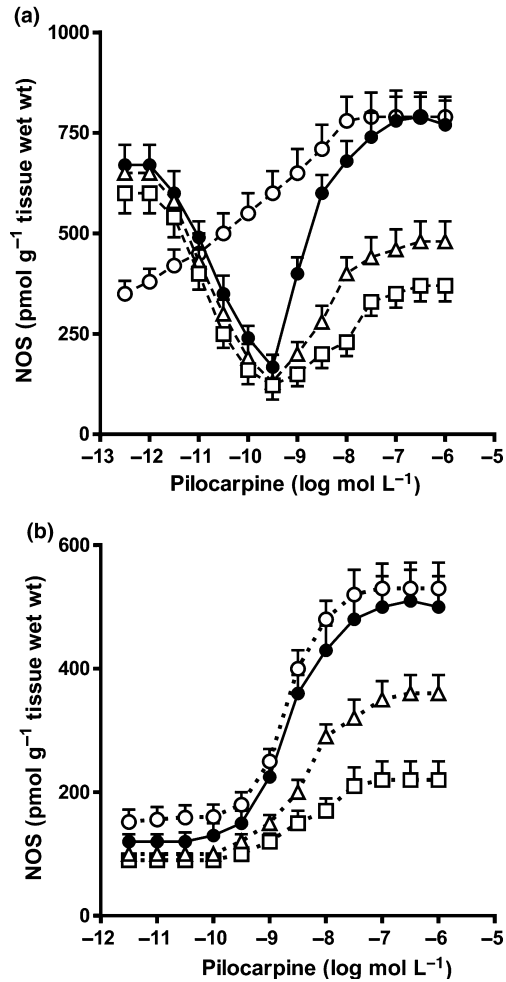


Figure 5 Effect of 1×10^{-6} mol L $^{-1}$ aminoguanidine (○), L-NIO 5×10^{-6} mol L $^{-1}$ (□) or 5×10^{-6} mol L $^{-1}$ NZ (Δ) on the concentration–response curve for the effect of pilocarpine (●) upon NOS activity of LPS-treated (a) or saline-treated (b) pulp. Data shown are the mean $\pm$ SEM of five experiments in each group, performed in duplicate.

parasympathetic activity may modulate the pulp inflammatory process was found, i.e. decreasing NO production by inhibiting iNOS activity and i-nos gene expression.

Previous findings, as well as this study showed that application of LPS to the pulp induced i-nos mRNA with a peak expression at 6 h, whilst i-nos mRNA expression was not observed in either saline-treated or untreated pulp (Kawashima *et al.* 2005). Moreover, it was observed that in saline-treated, as well as in healthy pulp, eNOS activity was identified as eNOS isoforms that were able to maintain pulpal basal

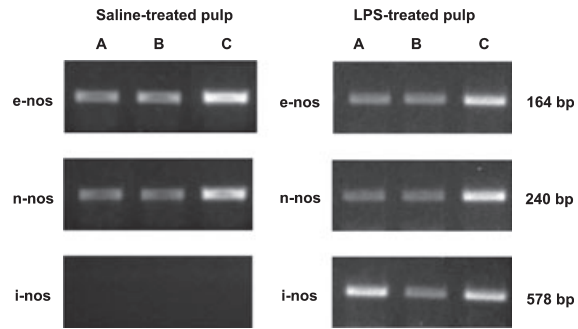


Figure 6 RT-PCR products for NOS isoforms: e-nos, n-nos and i-nos alone (a) or in the presence of pilocarpine 5×10^{-9} mol L $^{-1}$ (b) or 1×10^{-7} mol L $^{-1}$ (c), in saline-treated and LPS-treated pulp. Dental pulp was incubated for 1 h in KRB under identical experimental conditions as those described for NOS activity. Reaction products shown are one representative from five dental pulps tested in each group.

activity, whilst in LPS-treated pulp, the large basal NOS activity resulted in iNOS over-activity. These results agree with the concept that transition of the inflammatory process into irreversible pulpitis lowers e-nos level and increases the expression of i-nos (Di Nardo Di Maio *et al.* 2004). The large amount of NO produced by the significant up-regulation of iNOS activity may elicit negative feedback on eNOS activity in LPS-treated pulp. Indeed, the inhibition of iNOS activity markedly reduced basal NOS activity and NO production by LPS-treated pulp. Moreover, it was observed that the elevated level of NO in LPS-treated pulp retained a cytoplasmic localization, whilst in the saline-treated pulp, NO spread to the incubation medium. It is likely that these differences arise from the localization of eNOS and iNOS under normal and pathological conditions. Thus, eNOS is present in healthy pulp tissue in endothelial cells, fibroblasts and odontoblasts, whilst significant iNOS immunoreactivity is found mostly in the central area of inflamed pulp, especially near accumulated leukocytes (Di Nardo Di Maio *et al.* 2004).

However, the major finding of this study was that the progressive inflammation of the pulp caused a drastic change in the tissue response to mAChR activation. In LPS-treated pulp, pilocarpine at therapeutic concentrations decreased NOS activity and NO accumulation, whilst at high concentrations, the muscarinic agonist triggered a stimulatory response. On the contrary, in saline-treated and healthy pulp (Sterin-Borda *et al.* 2007), pilocarpine exerted only positive effect. The

alterations in functional regulation of mAChR activation observed in irreversible pulpitis may be caused by modification of i-nos induction in response to inflammatory stimuli. Thus, concentrations of pilocarpine that decreased NOS activity and NO accumulation also decreased basal i-nos mRNA level. On the other hand, the agonist at concentrations that increased NOS activity and NO production increased e-nos and n-nos mRNA levels, with a small increase in i-nos mRNA level. Moreover, the pilocarpine effect on LPS-treated pulp switched to a decrease from an increase when iNOS activity was inhibited by aminoguanidine. In contrast, the stimulatory effect of pilocarpine was reduced when eNOS and nNOS activity was inhibited. Although both eNOS and nNOS are constitutively expressed in pulp, mAChR agonist was more effective at activating eNOS than nNOS. Consequently, the nature of pilocarpine inhibition of NOS activity and NO accumulation suggests that an iNOS-mediated pathway may be relevant, whilst pilocarpine stimulation appears to be mediated by eNOS and nNOS pathways.

The paradoxical inhibitory effect of pilocarpine observed in LPS-treated pulp may be explained through a pharmacological phenomenon (Kenakin 2005), namely, constitutive receptor activity; that is, the ability of the receptor to produce signalling without exogenous agonist intervention. This spontaneously active state of some G protein coupled receptors might trigger elevated basal cellular activity that can be selectively inhibited by ligands (inverse agonism) (Berg *et al.* 2005). The constitutive activity of the receptor depends on the efficiency of coupling to transducing molecules (Gether *et al.* 1997). Thus, receptor mutation can change the receptor transducer coupling efficiency. If this is the case, in LPS-treated pulp, irreversible pulpitis may induce mAChR mutation. This increases the receptor transduction coupling efficiency to iNOS activity and i-nos gene expression, which leads to a spontaneously active conformation of the receptor. Therefore, pilocarpine action on constitutive mAChR activity may act as an inverse agonist and consequently produce an inhibitory effect upon NOS activity.

Conclusions

It should be noted that the association of the constitutive mAChR activity with irreversible pulpitis suggests that pilocarpine acting as an inverse agonist might be useful therapeutically to prevent necrosis and subsequent loss of dental pulp.

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References

- Berg KA, Harvey JA, Spampinato U, Clarke WP (2005) Physiological relevance of constitutive activity of 5-HT_{2A} and 5-HT_{2C} receptors. *Trends in Pharmacological Sciences* **26**, 625–30.
- Bergenholtz G, Nagaoka S, Jontell M (1991) Class II antigen expressing cells in experimentally induced pulpitis. *International Endodontic Journal* **24**, 8–14.
- Borda E, Furlan C, Orman B, Reina S, Sterin-Borda L (2007) Nitric oxide synthase and PGE₂ reciprocal interactions in rat dental pulp: cholinergic modulation. *Journal of Endodontics* **33**, 142–7.
- Carmignani M, Volpe A, Boscolo P (2000) Catecholamine and nitric oxide systems as targets of chronic lead exposure in inducing selective functional impairment. *Life Sciences* **68**, 401–15.
- Colasanti M, Suzuki H (2000) The dual personality of NO. *Trends in Pharmacological Sciences* **21**, 249–52.
- Dantzer R, Bluthé RM, Layé S, Bret-Dibat JL, Parnet P, Kelley KW (1998) Cytokines and sickness behaviour. *Annals of the New York Academy of Sciences* **840**, 586–90.
- Di Nardo Di Maio F, Lohinai Z, D'Arcangelo C *et al.* (2004) Nitric oxide synthase in healthy and inflamed human dental pulp. *Journal of Dental Research* **83**, 312–6.
- Edwall L, Olgart L, Haegerstam G (1973) Influence of vasodilator substances on pulpal blood flow in the cat. *Acta Odontologica Scandinavica* **31**, 289–96.
- Felaco M, Di Maio FD, De Fazio P *et al.* (2000) Localization of the e-NOS enzyme in endothelial cells and odontoblast of healthy human dental pulp. *Life Sciences* **68**, 297–306.
- Gardiner S, Compton A, Kemp P, Bennett T (1990) Regional and cardiac haemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats: effects of N-nitro-L-arginine methyl ester. *British Journal of Pharmacology* **105**, 429–35.
- Gether U, Ballesteros JA, Seifert R, Sanders-Bush E, Weinstein H, Kobilka BK (1997) Structural instability of a constitutively active G-protein coupled receptor agonist-independent activation due to conformational flexibility. *Journal of Biological Chemistry* **272**, 2587–90.
- Iijman T, Zhang J (2002) Three-dimensional wall structure and the innervation of dental pulp blood vessels. *Microscopy Research and Technique* **56**, 32–41.
- Kawanishi HN, Kawashima N, Suzuki N, Suda H, Takagi M (2004) Effects of an inducible nitric oxide synthase inhibitor

- on experimentally induced rat pulpitis. *European Journal of Oral Sciences* **112**, 332–7.
- Kawashima N, Kawanishi HN, Suzuki N, Takagi M, Suda H (2005) Effect of NOS inhibitor on cytokine and COX2 expression in rat pulpitis. *Journal of Dental Research* **84**, 762–7.
- Kenakin T (2005) The physiological significance of constitutive receptor activity. *Trend in Pharmacological Science* **26**, 603–5.
- Liu M, Kim S, Park D, Markowitz K, Bilotto G, Dorscher-Kim J (1990) Comparison of the effect of intra-arterial an locally applied vasoactive agent on pulpal blood flow in dog canine teeth determined by laser Doppler velocimetry. *Archives of Oral Biology* **35**, 405–10.
- Lohinai Z, Szekeley A, Benedek P, Csiliag A (1997) Nitric oxide synthase containing nerves in the cat and dog dental pulp and gingival. *Neuroscience Letters* **227**, 91–4.
- Luthman J, Luthman G, Höldelt T (1992) Occurrence and distribution of different neurochemical markers in the human dental pulp. *Archives of Oral Biology* **37**, 193–208.
- Maier SF, Goehler LE, Fleshner M, Watkins LR (1998) The role of the vagus nerve in cytokine-to-brain communication. *Annals of the New York Academy of Sciences* **840**, 289–300.
- Moilanen E, Whittle B, Moncada S (1999) Nitric oxide as a factor in inflammation. In: Gallin J, Snyderman R, eds. *Inflammation: Basic Principles and Clinical Correlates*. Philadelphia, PA: Lippincot Williams, Wilkins, pp. 367–72.
- Närhi M, Hirvonen T (1983) Functional changes in cat pulp nerve activity after thermal and mechanical injury of the pulp. *Proceedings of the Finnish Dental Society* **79**, 162–7.
- Okabe E, Todoki K, Ito H (1989) Direct pharmacological action of vasoactive substances on pulpal blood flow. An analysis and critique. *Journal of Endodontics* **15**, 473–7.
- Olgart L, Edwall L, Gazelius B (1991) Involvement of afferent nerves in pulpal blood-flow reactions in response to clinical and experimental procedures in the cat. *Archives of Oral Biology* **36**, 575–1.
- Olgart L, Kostouros GD, Edwall L (1996) Local actions of acetylcholine on vasomotor regulation in rat incisor pulp. *Acta Physiologica Scandinavica* **158**, 311–6.
- Photo P, Anttila R (1972) Innervation of blood vessels in the dental pulp. *International Dental Journal* **22**, 228–39.
- Sterin-Borda L, Gorelik G, Genaro A, Goin JC, Borda ES (1990) Human chagasic IgG interacting with lymphocyte neurotransmitter receptor triggers intracellular signal transduction. *FASEB Journal* **4**, 1661–7.
- Sterin-Borda L, Furlan C, Reina S, Orman B, Borda E (2007) Differential signalling pathways involved in cholinoceptor-dependent stimulation of nitric oxide isoforms in dental pulp. *International Endodontic Journal* **40**, 544–52.
- White D, Drew G, Gurden J, Penny D, Roach A, Watts I (1993) The effect of N-nitro-L-arginine-methyl ester upon basal blood flow and endothelium-dependent vasodilation in dog hindlimb. *British Journal of Pharmacology* **108**, 763–8.
- Yasuhara R, Suzawa T, Miyamoto Y et al. (2007) Nitric oxide in pulp cell growth, differentiation, and mineralization. *Journal of Dental Research* **86**, 163–8.

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