Annotating PDFs for eReturn

version 1.0; August 28, 2007; full release

1. Introduction

eProof files are self-contained PDF documents for viewing on-screen and for printing. They contain all appropriate formatting and fonts to ensure correct rendering on-screen and when printing hardcopy. DJS sends eProofs that can be viewed, annotated, and printed using the free version of Acrobat Reader 7 (or greater). These eProofs are "enabled" with commenting rights, therefore they can be modified by using special markup tools in Acrobat Reader that are not normally available unless using the Standard or Professional version.

The screen images in this document were captured on a PC running Adobe Acrobat Reader version 8.1.0. Though some of the images may differ in appearance from your platform/version, basic functionality remains similar. At the time of this writing, Acrobat Reader v8.1.0 is freely available and can be downloaded from: http://www.adobe.com/products/acrobat/readstep2.html

2. Comment & Markup toolbar functionality

A. Sticky Note tool; B. Text Edits tool; C. Stamp tool; D. Highlight Text tool; E. Callout tool; F. Text Box tool; G. Various Object tools; H. Pencil tool

A. Show the Comment & Markup toolbar

The Comment & Markup toolbar doesn't appear by default. Do one of the following:

- Select View > Toolbars > Comment & Markup.
- Select Tools > Comment & Markup > Show Comment & Markup Toolbar.
- Click the Review & Comment button in the Task toolbar, and choose Show Comment & Markup Toolbar.

To add or remove tools for this toolbar, right-click the toolbar and select the tool. Or, select Tools > Customize Toolbars.

B. Select a commenting or markup tool

Do one of the following:

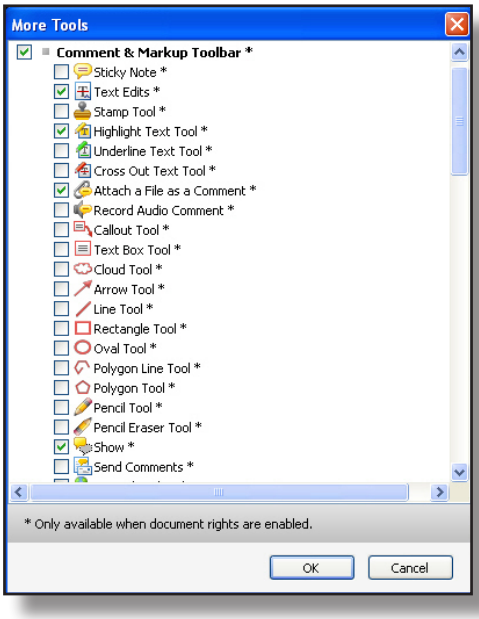
- Select a tool from the Comment & Markup toolbar.
- Select Tools > Comment & Markup > [tool].

Note: After an initial comment is made, the tool changes back to the Select tool so that the comment can be moved, resized, or edited. (The Pencil, Highlight Text, and Line tools stay selected.)

C. Keep a commenting tool selected

Multiple comments can be added without reselecting the tool. Select the tool to use (but don't use it yet).

- Select View > Toolbars > Properties Bar.
- Select Keep Tool Selected.



Choose Tools > Customize Toolbars to remove unnecessary items from the toolbar (see Section 7 for suggested toolbar layout)

3. The Properties bar

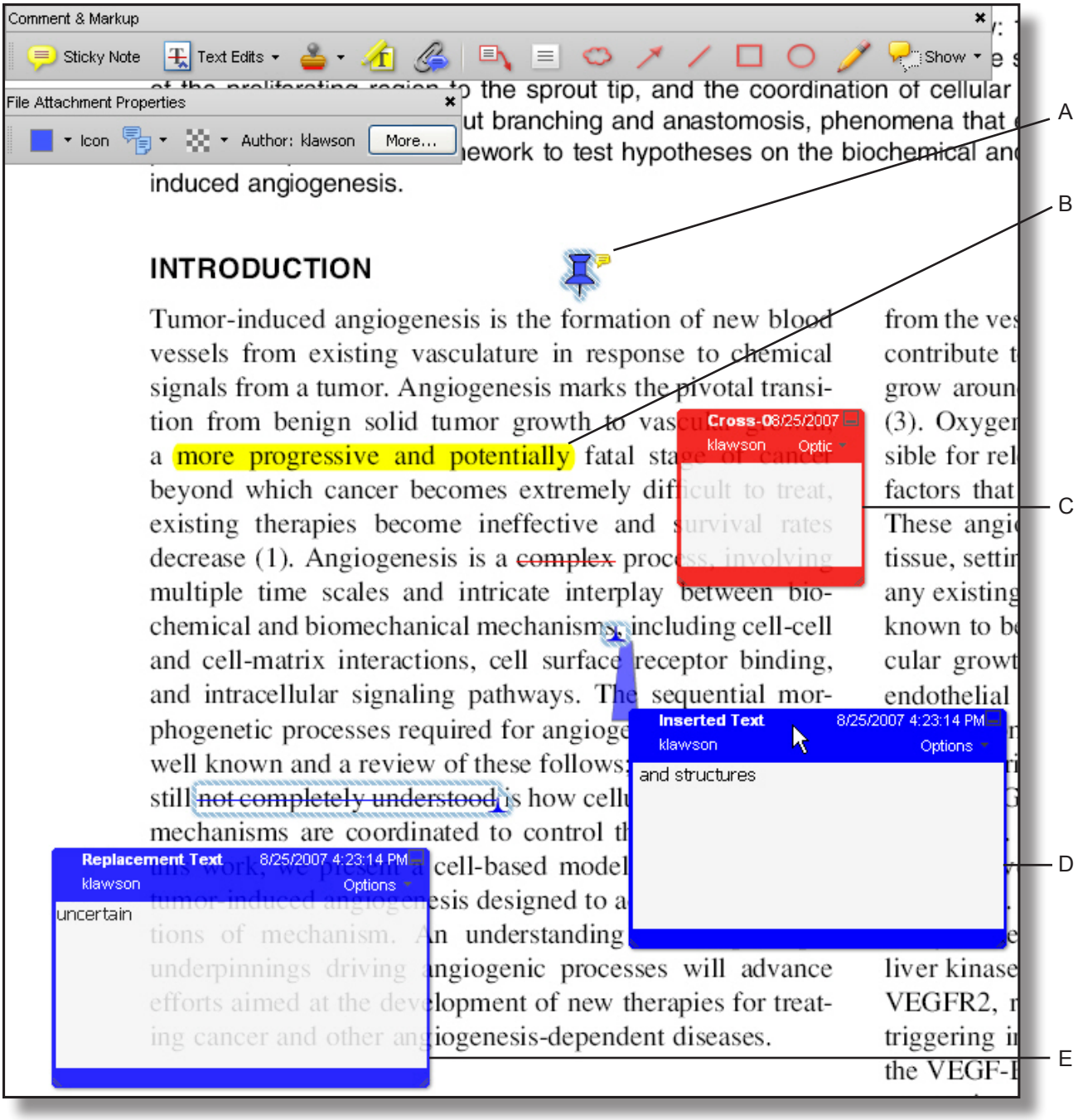
The Properties bar can be used to format text and select options for individual tools.

To view the Properties bar, do one of the following:

- Choose View > Toolbars > Properties Bar.
- Right-click the toolbar area; choose Properties Bar.
- Select [Ctrl-E]

4. Using the comment and markup tools

To *insert*, *delete*, or *replace* text, use the **Text Edits** tool. Select the Text Edits tool, then select the text with the cursor (or simply position it) and begin typing. A pop-up note will appear based upon the modification (e.g., inserted text, replacement text, etc.). Use the Properties bar to format text in pop-up notes. A pop-up note can be minimized by selecting the ⊟ button inside it.



A. Attached file; B. Highlighted text; C. Crossed-out (strike-through) text; D. Inserted text; E. Replaced text

5. Inserting symbols or special characters

An 'insert symbol' feature is not available for annotations, and copying/pasting symbols or non-keyboard characters from Microsoft Word does not always work. Use angle brackets < > to indicate these special characters (e.g., <alpha>, <beta>).

6. Editing near watermarks and hyperlinked text

eProof documents often contain watermarks and/or hyperlinked text. Selecting characters near these items can be difficult using the mouse alone. To edit an eProof which contains text in these areas, do the following:

- Without selecting the watermark or hyperlink, place the cursor near the area for editing.
- Use the arrow keys to move the cursor beside the text to be edited.
- Hold down the shift key while simultaneously using arrow keys to select the block of text, if necessary.
- Insert, replace, or delete text, as needed.

7. Summary of main functions

Insert text - Use Text Edits tool (position cursor and begin typing)
Replace text - Use Text Edits tool (select text and begin typing)
Delete text - Use Text Edits tool (select text and press delete key)
Highlight text - Use Highlight Text tool (select text)
Attach a file - Use the Attach a File with Comment tool (select tool, position cursor and click mouse, select file)

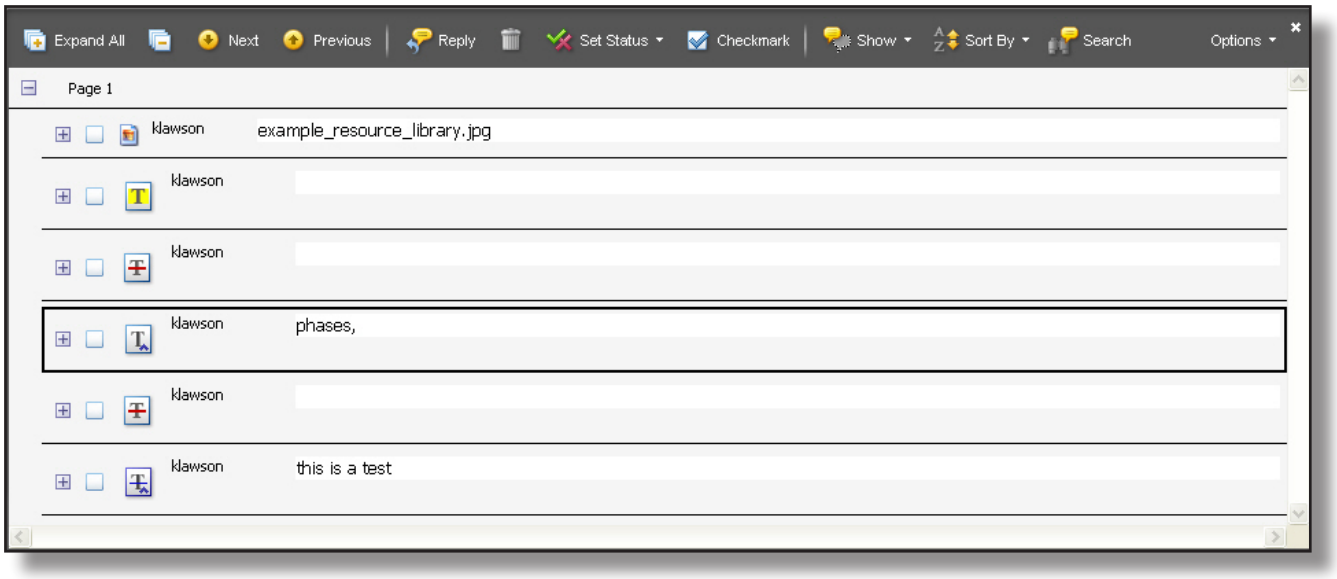
Suggested toolbar layout

8. Reviewing changes

To review all changes, do the following:

- Select the Show button on the Comment & Markup toolbar.
- Select Show Comments List.

Note: *Selecting a correction in the list will highlight the corresponding item in the document, and vice versa.*



Use the Comments list to review all changes

9. The eReturn process

A. An email is received that contains a link to the eProof of the article:
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B. Click on the link to open the proof with the internet browser. Select "Save As" from the browser's 'File' menu to save a copy of the PDF to the desktop or other folder.

C. Close the browser and open the saved PDF file with Acrobat.

D. Make corrections using Acrobat's Comment & Markup tools.

E. Save the PDF file, now with annotations, and return according to the instructions provided by the journal manager.

Cholinoceptor Modulation on Nitric Oxide Regulates Prostaglandin E_2 and Metalloproteinase-3 Production in Experimentally Induced Inflammation of Rat Dental Pulp

Alejandra De Couto Pita, Enri Borda,*† Sabrina Ganzinelli,*† Daniela Passafaro,* and Leonor Sterin-Borda*†*

Abstract

The purpose of this study was to investigate the role of muscarinic acetylcholine receptor (mAChR) activity in the regulation of inducible nitric oxide synthase (iNOS) activity, prostaglandin E_2 (PGE_2), and metalloproteinase-3 (MMP-3) in experimentally induced inflammation of rat incisors dental pulp. 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 were used as controls. NOS activity was measured by the production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine. PGE_2 and MMP-3 production were evaluated by an enzyme-linked immunosorbent assay (ELISA) and cyclooxygenase (cox-1 and cox-2) messenger RNA levels were measured using a reverse-transcriptase polymerase chain reaction by coamplification of target complementary DNA with a single set of primers. The application of LPS to the pulp increased NOS activity, PGE_2, and MMP-3 production associated with iNOS overactivity. Moreover, PGE_2 and MMP-3 production were the result of cox-2 expression. Pilocarpine (5×10^{-11} mol/L to 5×10^{-9} mol/L), acting on mAChRs, triggered a negative effect on NOS activity, PGE_2, and MMP-3 production. In control pulp, no action of pilocarpine was observed. Pulpitis changed mAChR conformation, increasing its coupling efficiency to transducing molecules that in turn activate iNOS. The capacity of pilocarpine to prevent iNOS activity, PGE_2, and MMP-3 by acting on mAChR mutation induced by pulpitis might be useful therapeutically as a local treatment. *(J Endod 2009;■:1–8)*

Key Words

Cyclooxygenase-1, cyclooxygenase-2, dental pulp, metalloproteinase-3, muscarinic acetylcholine receptor, nitric oxide synthase, pilocarpine, prostaglandin E_2, pulpitis

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Pulpal inflammation occurs as a consequence of bacterial infection resulting from caries, trauma, or iatrogenic causes (1). Pulpitis is an adaptative immune process against bacteria characterized by an increase in local blood flow (2), immunocompetent cell activation (3) with overproduction or imbalanced production of proinflammatory mediators (4), and alteration of neuronal activity (5). Thus, for controlling pulpal inflammatory reactions, it is essential not only that infection be eliminated but also that inflammation be controlled.

The parasympathetic system innervates lymphoid cells and tissues and can directly or indirectly influence the inflammatory reaction (2, 6). Muscarinic cholinergic activation may be involved in the composition and activation status of circulating immune cells by changing vascular tone or the local release of proinflammatory mediators (7, 8).

There is evidence for the presence of cholinergic nerves, muscarinic receptors, and acetylcholine-degrading enzymes in pulp tissue (9, 10). The parasympathetic innervation in the dental pulp is distributed around small blood vessels (11), and parasympathomimetic agents control the pulpal blood flow, which causes vasodilation (7, 12, 13). The vasodilatory action provoked by muscarinic cholinergic agonists has been shown to be dependent on the production of nitric oxide (NO) in a number of tissues (14, 15). Recently, we have shown the physiologic role of the parasympathetic system in healthy dental pulp and have established that the activation of muscarinic acetylcholine receptors (mAChRs) triggers the release of proinflammatory mediators, including NO and prostaglandin E_2 (PGE_2) (10, 16). However, the role of parasympathetic innervation in pulpitis has not been defined.

NO 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 by generating highly toxic compounds (17). Physiologic NO is synthesized by two constitutively expressed enzymes: endothelial (e) and neuronal (n) nitric oxide synthase (NOS). These isoforms of NOS can rapidly synthesize small amounts of NO for short periods of time after receptor stimulation in healthy dental pulp (16). nNOS is mainly localized in pulpal nerve fibers (18), and eNOS is detected in endothelial cells and odontoblasts of healthy dental pulp (19). On the other hand, detrimental NO is produced in response to inflammatory stimuli by inducible (i) NOS. iNOS produces large amounts of NO for prolonged periods (20), which can have a harmful effect on the immune response by acting as a toxic agent during infection, with subsequent abrasion of the tooth (21, 22). These properties of iNOS make it an essential and important mediator in pulpitis, especially if it is activated at the onset of inflammation.

Prostaglandins (PGs) are biologically active lipids synthesized from arachidonic acid, which, in turn, might generate NO (23). Some PGs and NO have been identified as mediators of inflammation (24, 25). PGE_2 has been strongly implicated in pulpal inflammation, particularly in vascular permeability and vasodilation. Experimental evidence suggests a relationship between PGs and NO biosynthesis. NO donors can stimulate or inhibit PGs biosynthesis in a variety of cellular systems. These effects could occur by alteration of cox gene transcription (26) by stimulation or inhibition of COX subtype activities or by modifying metabolic enzymes that convert PGs endoperoxides to stable eicosanoid products (27). The reciprocal interactions between PGE_2 and Q2

NO production and their physiologic role on healthy rat dental pulp have been established (10).

Matrix metalloproteinase-3 (MMP-3) belongs to the family of Zn endopeptidases that plays an important role in extracellular matrix turnover in which proteins, such as proteoglycan, fibronectin, elastin gelatin and collagen types II, III, IV, V, IX, X, and XI, are its substrates (28). Expression of MMP-3 in human gingival fibroblasts from healthy subjects and diseased patients has been shown (29), and levels of MMP-3 can differentiate between healthy and diseased periodontal sites (30). Moreover, the high MMP-3 levels in periodontal pockets can serve as a risk factor for periodontal disease progression (31). MMP-3 is secreted in response to inflammatory stimuli by gingival fibroblasts (28). However, the regulation of MMP-3 production in pulpal inflammation is poorly understood. The present study was undertaken to investigate the effect of NO and PGE_2 on MMP-3 secretion by experimentally induced inflammation of rat dental pulp. We also evaluated whether iNOS activity was related to the accumulation of NO and whether the muscarinic cholinergic agonist pilocarpine effectively regulated iNOS activity, PGE_2, and MMP-3 production during pulpitis. The results may contribute to understanding modulation of the parasympathetic system in the course of chronic pulpitis and suggest that mAChR agonists may be useful as a local treatment to decrease pulpal inflammation.

Materials and Methods

Animals

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

Induction of Pulpitis

Under general anesthesia induced with intramuscular ketamine (62.5 mg/kg) and intramuscular ~~Xylocaine,~~ the pulp of the left and right upper 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 #40, in order to create sufficient space to apply lipopolysaccharide (LPS) or saline. LPS from *Escherichia coli* O111:B4 (Sigma Chemical Co, St Louis, MO) was dissolved in sterile saline at a concentration of 10 mg/mL, 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 anesthesia at 6 hours after pulp exposure, and the incisors were extracted. LPS application causes maximal inflammatory reaction in the coronal area of the pulp at 6 hours and decreased thereafter confirming previous report (4). Findings were characterized by the 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 hours, which confirmed previous studies (4, 32). Fresh dental pulp tissue was kept at room temperature in Krebs Ringer bicarbonate (KRB) solution in the presence of 5% CO_2 in oxygen until the various experimental assays were performed. Nontreated healthy rat dental pulp tissue from the upper incisors (left and right) and treated with sterile saline were used as controls.

Determination of NOS Activity

NOS activity was measured in rat dental pulp tissue by the production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine as previously described (10). Briefly, pulp was incubated for 30 minutes in 500 μL KRB solution that contained 18.5 KBq of L-[U-^{14}C]-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 minutes before the end of incubation. Incubation was performed in a 5% CO_2 in oxygen atmosphere at 37°C. Tissues were then homogenized in an Ultra Turrax homogenizer in 500 μL of medium that contained 20 mmol/L ~~HEPES~~, pH 7.4, 0.5 mmol/L ~~EGTA~~, 0.5 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L leupeptin, and 0.2 mmol/L phenylmethylsulfonyl fluoride at 4°C. Supernatants were applied to 2-mL columns of Dowex AG 50WX-8 (sodium form), and [^{14}C]-citrulline was eluted with 3 mL water and quantified by liquid scintillation counting (Beckman LS 6500, New York, NY).

PGE_2 and Assays

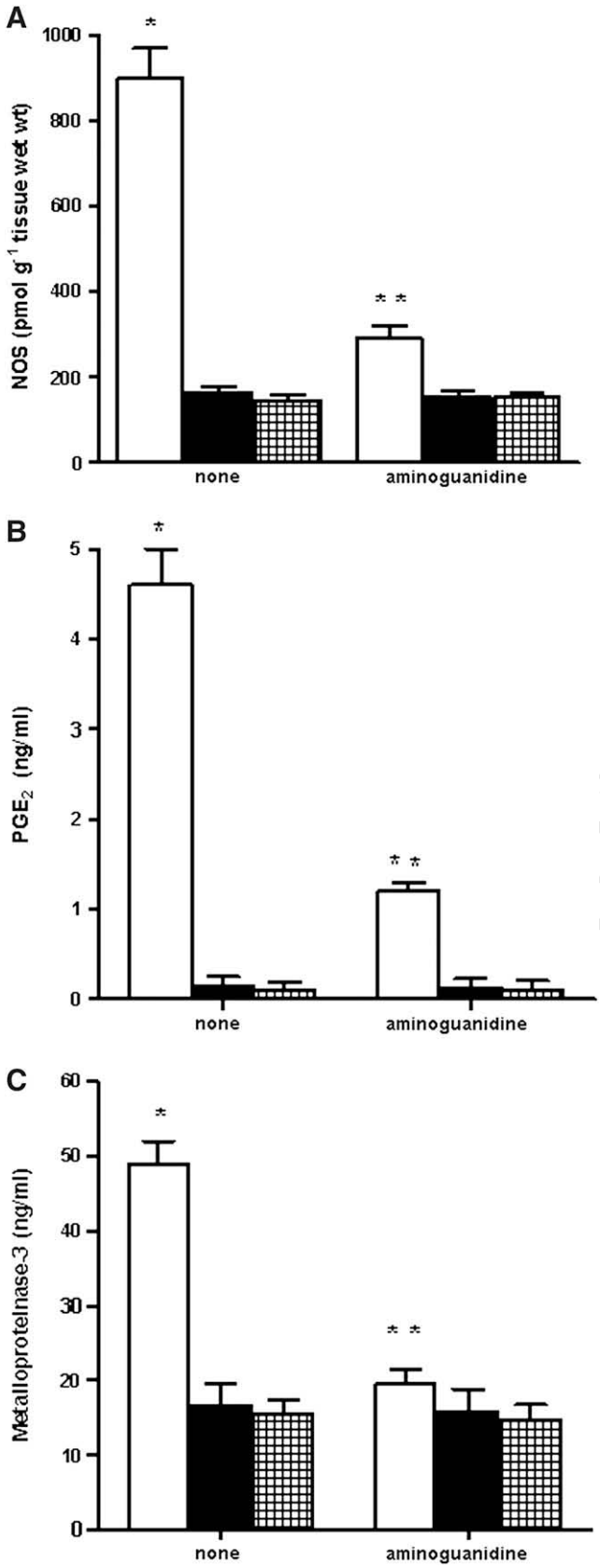
Rat dental pulp (10 mg) was incubated for 60 minutes in 0.50 mL of KRB gassed with 5% CO_2 in oxygen at 37°C. Pilocarpine was added 30 minutes before the end of incubation period and blockers 30 minutes before the addition of different pilocarpine concentrations. Dental pulp was then homogenized into a 1.5-mL polypropylene microcentrifuge tube. Thereafter, all procedures used were those indicated in the protocol of PGE_2 Biotrak Enzyme Immuno Assay System (Amersham Biosciences, Piscataway, NJ). For the extraction of extracellular matrix components, the method described by Talhouk et al (33) was used with minor modifications. Briefly, samples were mixed with extraction buffer (0.5 mol/L Tris HCl [pH 7.5], 1% Triton X-100, 10 mmol/L $CaCl_2$, and 200 mmol/L NaCl) in a ratio of 1:5 (weight/volume) at 4°C and homogenized in a glass/glass conical homogenizer. The homogenate was then subjected to three freeze-thaw cycles of 5 minutes each and centrifuged at 13,000*g* for 30 minutes at 4°C. The detergent-soluble supernatant was recovered and stored at −70°C for further analysis, and the insoluble pellet fractions were discarded. Protein determination was performed by Lowry et al (34). ~~Detergent extracts~~ of pulps were analyzed by the Enzyme Immuno Assay System to determine the enzymatic activity of MMP-3 according to manufacturer's instructions (Amersham Matrix Metalloproteinase-3) (Biotrak Activity Assay System; GE Healthcare, NJ). The PGE_2 and MMP-3 results were expressed as nanograms per milliliter.

Messenger RNA Isolation and Complementary DNA Synthesis

Total RNA was extracted from rat dental pulp tissue by homogenization using the guanidinium isothiocyanate method as previously described (16): a 20-μL reaction mixture that contained 2 ng messenger RNA (mRNA), 20 U RNase inhibitor, 1 mmol/L ~~dNTPs~~, and 50 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). First-strand complementary DNA (cDNA) was synthesized at 37°C for 60 minutes.

Polymerase Chain Reaction Procedures

Cox isoform mRNA levels were determined by a method that involved simultaneous coamplification of both the target cDNA and a reference template (MIMIC) with a single set of primers. MIMIC for cox-1 and cox-2 and glyceraldehyde-3-phosphate dehydrogenase (g3pdh) was constructed using a polymerase chain reaction (PCR) MIMIC construction kit (Clontech Laboratories, Palo Alto, CA). 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 construction of MIMIC and amplification of cox isoforms and g3pdh mRNA were as reported previously (35). 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 $MgCl_2$, 0.4 μmol/L primer, dNTPs, 2.5 U Taq DNA polymerase, and 0.056 μM Taq Start antibody (Clontech Laboratories). After the initial denaturation at 94°C for 2 minutes, the cycle conditions were 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and 45 seconds for enzymatic primer extension at 72°C for 45 cycles 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 minutes followed by 30 cycles of amplification. Each cycle consisted of 35 seconds at 94°C, 35 seconds at 58°C, and 45 seconds at 7°C. Samples were incubated for an additional 8 minutes at 72°C before completion. PCR products were subjected to electrophoresis following previously described procedures (35). Different cox 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-Aldrich Chemical Company (St Louis, MO); 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA); l-[4,5-bis(4-methoxyphenyl)-2-thiazolyl]carbonil-4-methylpiperazine hydrochloride (FR 122047) and 5-bromo-2-(4-urophenyl)-3-[4-(methyl sulfonyl) phenyl]-thiophene (DuP 697) were obtained from Tocris Cookson Inc (Ellisville, MO). Stock solutions were freshly prepared in the corresponding buffer.

Statistical Analysis

A Student *t* test for unpaired values was used to determine the levels of significance.

Analysis of variance and the Student-Newman-Keuls test were used when pair-wise multiple comparison procedures were necessary. Differences between mean values were considered significant at $p < 0.05$.

Results

Figure 1 shows that basal values of NOS activity, PGE_2, and MMP-3 production in LPS-treated pulp at 6 hours were significantly higher than those of saline-treated or untreated pulp tissues. To show if iNOS isoforms might be implicated in the increase of NOS activity and in the production of PGE_2 and MMP-3 in LPS-treated pulp, isolated rat dental pulp tissue was incubated with a specific inhibitor of iNOS isoforms. As can be seen in Figure 1, the inhibition of iNOS by aminoguanidine (1×10^{-6} mol/L) significantly decreased the basal activity of NOS and the

Figure 1. (*A*) Basal NOS activity (A), (*B*) PGE_2, and (*C*) MMP-3 in LPS-treated (□), saline-treated (■) and untreated pulp, without isoforms enzyme inhibitors (none) or in the presence of aminoguanidine (1×10^{-6} mol/L). Tissues were incubated for 30 minutes with or without drugs before the assays were performed. Data shown are absolute basal values and represent the mean ± standard error of the mean of six experiments in each group performed in duplicate. *$p < 0.0001$ versus saline-treated or untreated pulp. **$p < 0.005$ versus LPS-treated pulp without aminoguanidine.

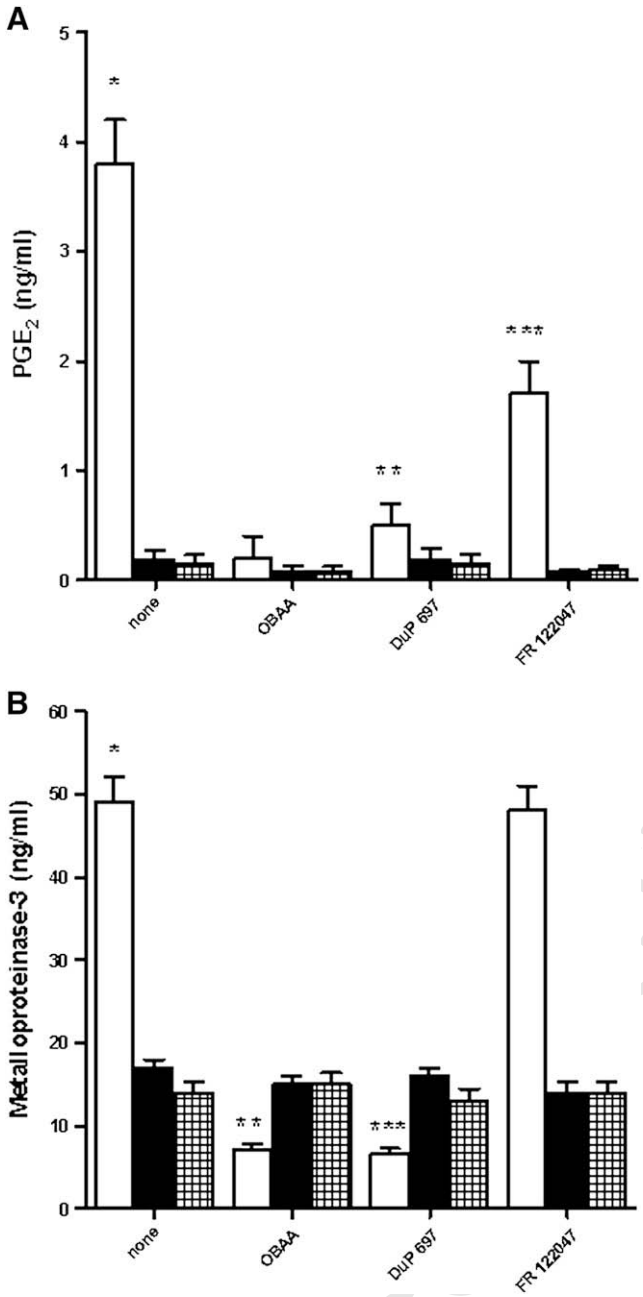


Figure 2. Basal, (*A*) PGE_2, and (*B*) MMP-3 values in LPS-treated (□), saline-treated (■), and untreated (▤) pulp, without enzyme inhibitors (none) or in the presence of OBAA (5×10^{-6} M) or DuP 697 (5×10^{-8} M) or FR 122047 (5×10^{-8} M). Data shown are the absolute basal values and represent the mean ± standard error of the mean of seven experiments in each group performed in duplicate. *$p < 0.0001$ versus saline-treated or untreated pulp. **$p < 0.001$ versus LPS-treated pulp without OBAA or DuP 697. ***$p < 0.005$ versus LPS-treated pulp without FR 122047.

production of PGE_2 and MMP-3 in LPS-treated pulp but had no effect on those from saline-treated and healthy pulp.

This observation strengthened the causal relationship between the increase in iNOS activity and the production of PGE_2 and MMP-3 in LPS treatment of pulp, indicating that the high production of PGE_2 and MMP-3 observed in LPS-treated pulp could be the result of an elevated level of NO generated by iNOS activation.

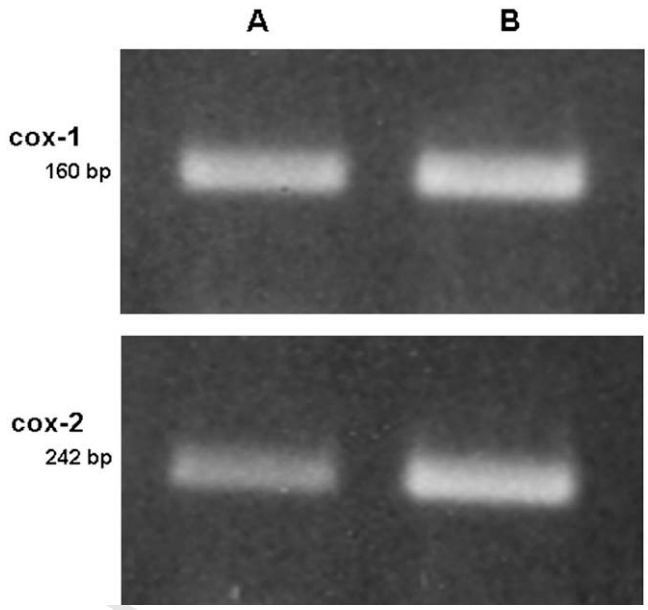


Figure 3. RT-PCR products for cox-1 and cox-2 isoforms obtained from (*A*) saline-treated pulp or (*B*) LPS-treated pulp. Dental pulp was obtained and prepared as described in the Material and Methods section, and then mRNA synthesis was extracted and determined. Reaction products shown are one representative from six dental pulps tested in each group.

To determine whether PGE_2 and MMP-3 generation are dependent on each other, dental pulp preparations were incubated with different inhibitors of the enzymatic pathways known to be involved in PGE_2 production. Figure 2 shows that the inhibition of phospholipase A_2 (PLA_2) by OBAA (5×10^{-6} mol/L) and COX-2 by DuP 697 (1×10^{-6} mol/L) significantly decreased the high basal values of both PGE_2 and MMP-3 production observed in LPS-treated pulp. However, the inhibition of COX-1 by FR 122047 (1×10^{-6} mol/L) decreased the high basal values of PGE_2 without an effect on MMP-3 production. As a control, Figure 2 also shows that all the enzymatic blockers had no effect on PGE_2 and MMP-3 generation from saline-treated pulp and healthy pulp.

To confirm the role of COX isoforms on LPS-treated pulp, reverse-transcription PCR was used on cox-1 and cox-2 mRNA obtained from LPS- and saline-treated pulp. Using specific oligonucleotide primers, RT-PCR amplified products (Figure. 3) showed bands of the predicted size for cox-1 and cox-2 detected in dental pulp. However, although high levels of cox-2 mRNA were detected only in LPS-treated pulp, the level of cox-1 mRNA was similar in LPS-treated pulp and in controls.

We have previously shown that pilocarpine, through activation of mAChR, increased levels of NO and PGE_2, modulating their own release by affecting constitutive NOS and COX activities, respectively, in healthy rat dental pulp. In the present study, we evaluated whether pilocarpine can modulate the high levels of NOS activity and the production of PGE_2 and MMP-3 in LPS-treated pulp.

Figure 4 shows the effect of increasing concentrations of pilocarpine on LPS-treated compared with saline-treated pulp and nontreated pulp. It can be seen that in LPS-treated pulp, pilocarpine decreased NOS activity, PGE_2, and MMP-3 production, which reached a maximum inhibition at a concentration of 5×10^{-9} mol/L, whereas at the same concentration range, the mAChR agonist had no effect in saline-treated pulp or nontreated pulp. Table 1 shows that the pilocarpine negative effects were abolished by atropine (5×10^{-7} mol/L) and was reversed by aminoguanidine (1×10^{-6} mol/L). Figure 5 showed that under

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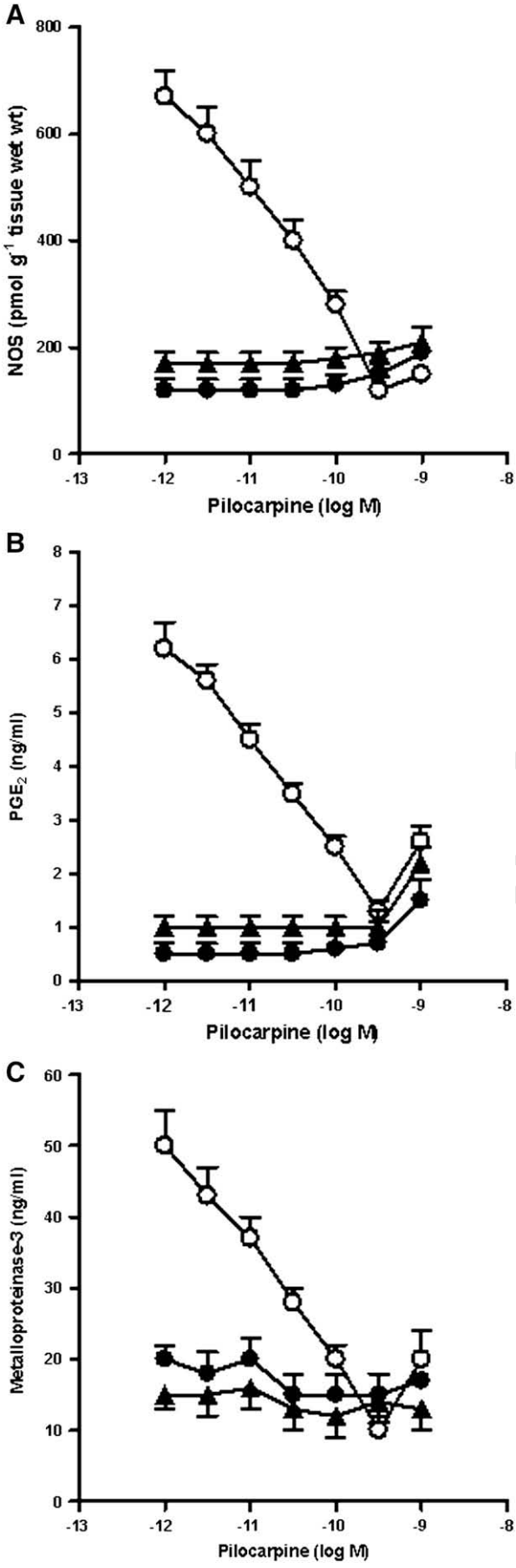

TABLE 1. Action of Different Blockers on Pilocarpine-Induced Negative effect on LPS-Treated Pulp

Conditions	NOS	PGE_2	MMP-3
Basal	756 ± 65	6.8 ± 0.52	53.3 ± 4.8
Pilocarpine	102 ± 10*	1.3 ± 0.08*	8.9 ± 0.9*
Pilocarpine +atropine	692 ± 58	6.4 ± 0.53	50.6 ± 5.1
Pilocarpine +aminoguanidine	735 ± 71	5.9 ± 0.49	49.4 ± 4.2

Values are mean ± standard error of the mean of five experiments in each group performed by duplicated. Data represent the effect of 5×10^{-9} mol/L pilocarpine that triggered the maximal inhibitory effect. NOS activity is expressed in picomole per gram of tissue wet weight and PGE_2 and MMP-3 in nanograms per microliter. They were measured incubated LPS-treated pulp with or without inhibitors for 40 minutes; pilocarpine was added in the last 10 minutes. The final concentrations of atropine was 1×10^{-7} mol/L and of aminoguanidine 1×10^{-6} mol/L .

*$p < 0.0001$ versus basal.

identical experimental conditions, a significant correlation (correlation coefficient = 0.05) between pilocarpine inhibited NOS activity, PGE_2, and MMP-3 generation was found. These results indicate that the mAChR agonist decreased PGE_2 and MMP-3 generation as a result of the decrease in NOS activity in LPS-treated pulp.

To further characterize the role of mAChR agonist on LPS-treated pulp, pilocarpine action on NOS activity was evaluated in in vivo treatment. Figure 6 (upper panel) shows comparatively the in vivo and in vitro effects of different pilocarpine doses on NOS activity. It can be seen that in LPS-treated pulp at 6 hours, the dose-response curves of pilocarpine to decrease the NOS activity were similar with in vivo and in vitro treatments. Figure 6 (lower panel) shows the decrement of NOS activity when a single dose of pilocarpine (5×10^{-9} mol/L) was administered in vivo to LPS-treated pulp at different times of exposure. The inhibitory effect of 5×10^{-9} mol/L pilocarpine on NOS activity varied according to the interval between which LPS and pilocarpine were applied. Thus, the inhibitory effect of pilocarpine was maximal when it was applied together with LPS (0 minutes), and the effect was minimal when the mAChR agonist was applied 5.5 hours (330 minutes) after LPS treatment.

Discussion

The role of the parasympathetic system in modulating the inflamed pulp is still unknown. In this study, we found pharmacologic evidence by which muscarinic parasympathetic receptor activity may modulate the pulp inflammatory process, decreasing NOS activity. Moreover, we reveal here that the application of LPS to the pulp induced increased production of PGE_2 and MMP-3 associated with high activity of NOS.

The increased production of PGE_2 and MMP-3 and the overactivity of NOS were dependent on iNOS because they were blocked by aminoguanidine at concentrations known to inhibit iNOS activity. Previous findings showed that in LPS-treated pulp there is an induction of i-nos mRNA with a peak expression at 6 hours, whereas i-nos mRNA expression was not observed in either saline-treated or untreated pulp (4). These results agree with the concept that the

Figure 4. The concentration-response curve of pilocarpine on (*A*) NOS activity, (*B*) PGE_2, and (*C*) MMP-3 in the LPS-treated pulp (○) compared with the response of saline-treated pulp (●) or untreated pulp (▲). Data shown are the mean ± standard error of the mean of six experiments in each group performed in duplicate; $p < 0.0001$ between LPS-treated pulp and both the saline-treated pulp or untreated pulp. See also the results referred to Table 1.

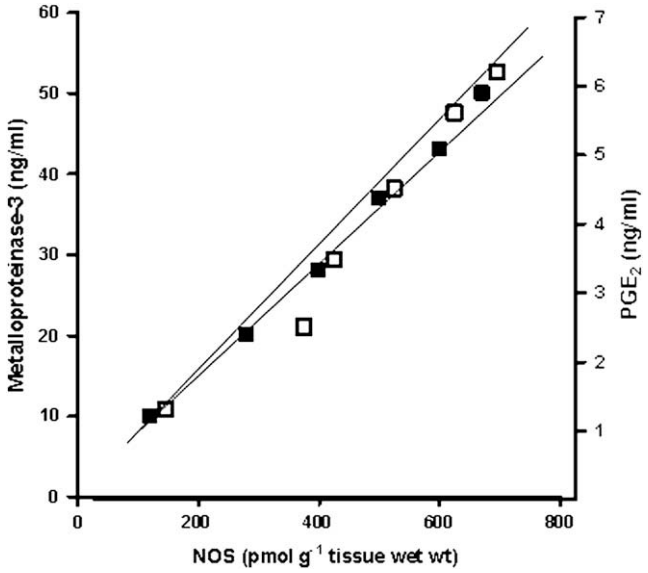

Figure 5. The correlation in the inhibitory effect of pilocarpine on NOS activity, PGE_2, and MMP-3 in the LPS-treated pulp. NOS activity was plotted as a function of PGE_2 (■) (Pearson $r = 0.9847$, $p < 0.0003$) or MMP-3 (□) (Pearson $r = 0.9977$, $p < 0.0001$). Data shown are the mean ± standard error of the mean of six experiments in each group performed in duplicate.

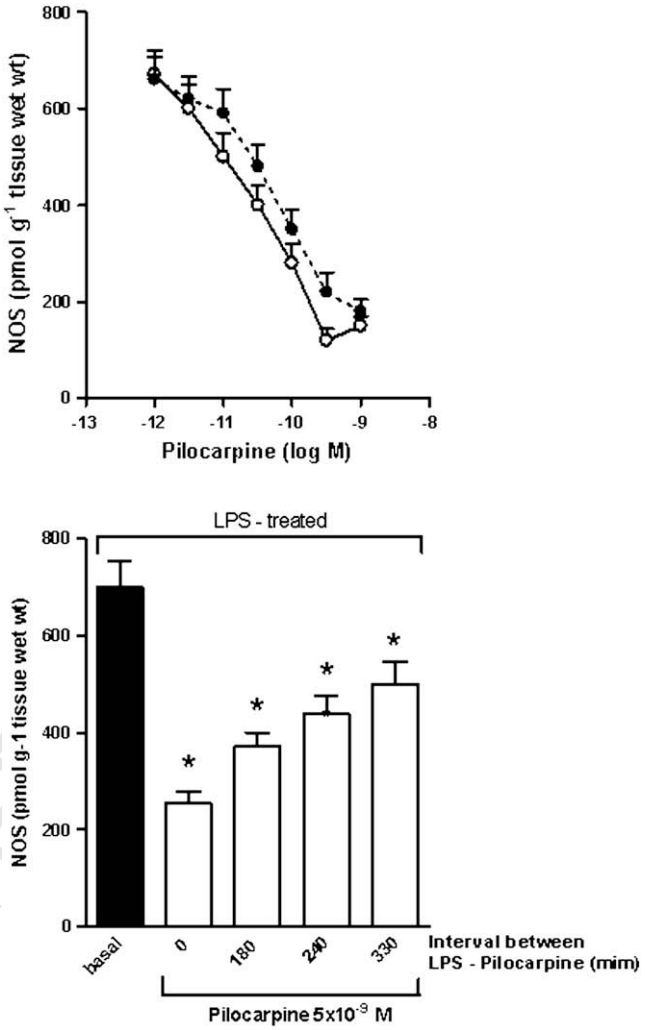

Figure 6. (*Upper panel*) Comparison between the in vitro (○) and in vivo (●) concentration-response curve of pilocarpine on NOS activity in LPS-treated pulp at 6 hours. Data shown are the mean ± standard error of the mean of six experiments in each group performed in duplicate. (Lower panel) In vivo kinetic response of pilocarpine on NOS activity. Pilocarpine at 5×10^{-9} mol/L was applied to the pulp together with LPS (0 minutes) or after 180 minutes, 240 minutes, or 330 minutes that LPS was applied. Values are mean ± standard error of the mean of five experiments in each group. *$p < 0.05$ versus LPS alone (basal).

transition of the inflammatory process into irreversible pulpitis lowers the constitutive nos level and increases expression of i-nos (36). Moreover, we previously reported that in healthy pulp, eNOS activity was identified as NOS isoforms that were able to maintain pulpal basal activity (16).

The large amount of NO produced by the significant upregulation of iNOS activity may elicit an increased production of PGE_2 and MMP-3 in LPS-treated pulp. Indeed, we showed in this article that the inhibition of iNOS activity markedly reduced basal PGE_2 and MMP-3 by LPS-treated pulp. In accordance with this, it has been shown that once iNOS is induced, it can produce copious quantities of NO for a prolonged period (20), which further affects the synthesis of the chemical mediators, such as PGs (36).

Prostaglandins and NO represent some of the most relevant local mediators that participate under basal conditions in the modulation of many cellular functions. In vivo studies have shown that the NO-dependent pathway plays an important role in regulating the basal vasodilator tone and blood circulation in dental pulp (18). The biosynthesis and release of NO and prostaglandins share a number of similarities. Under normal circumstances, the constitutive isoforms of these enzymes (constitutive NOS and COX-1) are found in virtually all organs. On the other hand, in inflammatory settings, the inducible isoforms of these enzymes (iNOS and COX-2) are detected (20). Evidence indicates that cross-talk between NO and prostaglandins in dental pulp exists (10). An important link between NOS and COX pathways has been shown here, raising the possibility that COX enzymes represent important endogenous "receptor" targets for modulating the multifaceted roles of NO. Furthermore, a different regulation of NO- activated COX isoforms to induce PGE_2 production between healthy or saline-treated pulp and LPS-treated pulp has been shown. Analysis of the data suggests that in LPS-treated pulp, the NO-induced PGE_2 is the result of both COX-1 and COX-2 activities, whereas in control pulp it is only associated with COX-1 activity. Previous findings (4), as well as our own data, clearly showed that the application of LPS to the pulp induced cox-2 mRNA expression at 6 hours. Moreover, the inhibition of NOS dramatically inhibited cox-2 expression in pulpal inflammation (4).

The heterogeneity of inflammatory responses in healthy and diseased pulp could also be reflected by the evidence for a differentially functional link between PGE_2 generation and MMP-3 production in pulp derived from LPS-treated or controls. The mechanism by which PGE_2-induced stimulation of MMP-3 production on LPS-treated pulp appears to occur secondarily to the activation of a number of enzymatic pathways commonly associated with PGE_2 biosynthesis. Thus, phospholipase A_2 and COX-2 inhibitors abrogated the high basal level of MMP-3 observed in LPS-treated pulp, whereas in either saline-treated or untreated pulp the MMP-3 production was not modified by the inhibitors. The fact that the inhibition of COX-1 did not affect MMP-3 production could indicate that the overproduction of MMP-3 is related to the expression of proinflammatory enzymes. From these data, we suggest

that NO induced cox-2 expression in LPS-treated pulp and the PGE_2 produced by COX-2 induced MMP-3 production. The stimulatory effect of PGE_2 on MMP-3 secretion in oral inflammatory disease has been described (28).

However, the major finding of the present study was that progressive inflammation of the pulp caused a drastic change in the tissue response to mAChR activation. In LPS-treated pulp, pilocarpine decreased NOS activity at therapeutic concentrations, with accumulation of PGE_2 and MMP-3. In contrast, pilocarpine did not exert any effect in saline-treated and healthy pulp at the same concentration.

The alterations in functional regulation of mAChR activation observed in pulpitis may be caused by modification of i-nos induction in response to inflammatory stimuli. Thus, the inhibition of iNOS activity by aminoguanidine reversed the inhibitory action of pilocarpine on NOS activity, PGE_2, and MMP-3. Also, the concentrations of pilocarpine that decreased NOS activity, PGE_2, and MMP-3 also decreased basal NOS activity. Moreover, a significant correlation between pilocarpine-inhibited NOS activity, PGE_2, and MMP-3 production was shown.

The paradoxic inhibitory effect of pilocarpine observed in LPS-treated pulp may be explained through a pharmacologic phenomenon, namely constitutive receptor activity (ie, the ability of the receptor to produce signaling 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. The constitutive activity of the receptor depends on the efficiency of coupling to transducing molecules (37). Thus, receptor mutation can change the receptor transducer coupling efficiency. If this is the case, in LPS-treated pulp, pulpitis may induce mAChR mutations. 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 on NOS activity, PGE_2, and MMP-3 production.

The downregulation of NOS activity triggered by pilocarpine in LPS-treated pulp was observed when the mAChR agonist was applied, even when obtained in vivo and in vitro with the same efficiency and potency in the dose-response curves. When we evaluated the kinetics of the pilocarpine-therapeutic effect, it was shown that the drug was more effective when more was applied to the beginning of pulpal inflammation. This is a very important issue because the expression of proinflammatory cytokines and cox-2 began or increased 3 hours after the application of LPS, corresponding to the onset of pulpitis (4). Considering the properties of NO that could induce chemical mediators, the blockade of NO synthesis by pilocarpine could be responsible for the downregulation of proinflammatory substances and cox-2 expression that are largely produced by inflammatory cells (32). If this is the case, pilocarpine could behave as a selective inhibitor of iNOS that downregulated the chemokine expression during inflammation (38) and was effective for inhibiting the infiltration of inflammatory cells into pulp (32).

In conclusion, iNOS activity may be related to the initiation of mediator production, such as NO, PGE_2, and MMP-3. NO/PGE_2 would also cause severe relaxation of blood vessel smooth muscles and enhancement of permeability in inflamed pulp. The muscarinic agonist, pilocarpine, inhibited overproduction of NO, and, therefore, the phenomena might be harmful to low compliant pulp tissue. Thus, the administration of low pilocarpine doses may have a beneficial action on pulp inflammation to prevent necrosis and subsequent loss of dental pulp. Figure 7 shows a schematic illustration of the proposed mechanism between mAChRs and iNOS, PGE_2, and MMP-3 in LPS-treated pulp and how the Muscarinic agonist pilocarpine inhibited the overproduction of NO and could be useful as a therapeutic agent to decrease pulpal inflammation.

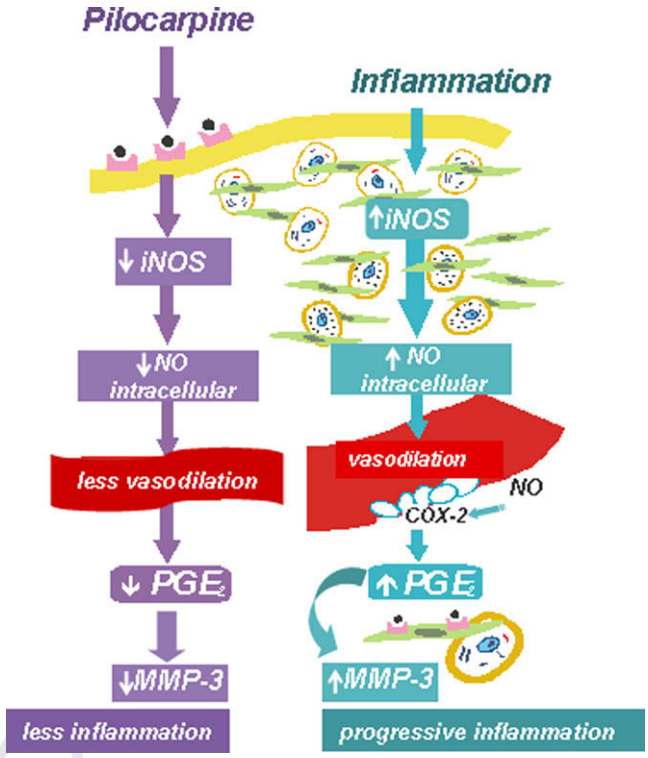

Figure 7. A proposed model for a mechanism in which pilocarpine downregulates the NO/PGE_2 MMP-3 pathway on pulpal inflammation. Inflammation triggers the induction and activation of iNOS by the endothelium blood vessels, fibroblasts, and macrophages, which, in turn, catalyzes NO synthesis. The NO diffuse into the smooth muscle where it mediates vasodilatation. On the other hand, NO induce the expression of cox-2 mRNA level causing the release of PGE_2, which also maintained the vasodilatation. MMP-3 is secreted in response to the PGE_2 accumulation. The activation of mAChR by pilocarpine on endothelium blood vessels fibroblasts and macrophages inhibits the inflammation-induced iNOS activation causing a decrease in overproduction of NO that, in turn, prevents severe relaxation PGE_2 release and MMP-3 production, decreasing pulpal inflammation.

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