British Journal of Pharmacology (1998) 123, 353–360

Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat

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1 Excessive production of nitric oxide (NO), and the generation of peroxynitrite have been implicated in various proinflammatory conditions. In the present study, using mercaptoethylguanidine (MEG), a selective inhibitor of iNOS and a peroxynitrite scavenger, we investigated the role of iNOS and peroxynitrite in a rat model of periodontitis.

2 Periodontitis was produced in rat by a ligature of 2/0 braided silk placed around the cervix of the lower left 1st molar. Animals were then divided into two groups: one group of rats was treated with MEG (30 mg kg^{-1}, i.p., 4 times per day for 8 days), animals in the other group received vehicle. At day 8, the gingivomucosal tissue encircling the mandibular 1st molars was removed on both sides from ligated and sham operated animals for inducible nitric oxide synthase (iNOS) activity assay and for immunocytochemistry with anti-iNOS serum. Plasma extravasation was measured with the Evans blue technique. Alveolar bone loss was measured with a videomicroscopy.

3 Ligation caused a significant, more than 3 fold increase in the gingival iNOS activity, whereas it did not affect iNOS activity on the contralateral side, when compared to sham-operated animals. Immunohistochemical analysis revealed iNOS-positive macrophages, lymphocytes and PMNs in the connective tissue and immunoreactive basal layers of epithelium on side of the ligature, and only a few iNOS-negative connective tissue cells on the contralateral side. Ligation significantly increased Evans blue extravasation in gingivomucosal tissue and alveolar bone destruction compared to the contralateral side. MEG treatment significantly reduced the plasma extravasation and bone destruction.

4 The present results demonstrated that ligature-induced periodontitis increases local NO production and that MEG treatment protects against the associated extravasation and bone destruction. Based on the present data, we propose that enhanced formation of NO and peroxynitrite plays a significant role in the pathogenesis of periodontitis.

Keywords: Ligature; inducible nitric oxide synthase; nitric oxide; periodontitis; gingivitis; lipopolysaccharide; cytokine; tissue destruction

Introduction

Nitric oxide (NO) is a free radical with important cardiovascular, neurological and immune functions. NO is produced by a group of isoenzymes collectively termed NO synthases (NOS). NOS exists as three distinct isoforms, namely endothelial NOS (eNOS), brain NOS (bNOS) and inducible NOS (iNOS). The eNOS and bNOS release small amounts of NO for a short period of time following receptor stimulation. In contrast iNOS is expressed in response to proinflammatory stimuli (endotoxin of Gram-negative bacteria alone or together with certain cytokines) and produces a large amount of NO for sustained periods of time. NO produced in high local concentration may act as a cytotoxic molecule acting against fungal, bacterial, protozoal organisms as well as tumour cells (for review see: Moncada, 1992; Wong & Billiar, 1995; Southan & Szabó, 1996). Recent data indicated that NO, independently, may be less toxic than it has originally been proposed, and the formation of peroxynitrite (a toxic product of NO when combining with superoxide) may be responsible for much of the NO-related cytotoxicity (Beckman & Koppenol, 1996; Szabó, 1996).

Excessive NO or peroxynitrite formation has been implicated in the pathophysiology of various inflammatory conditions, including gastrointestinal diseases such as ulcerative colitis and ileitis (Boughton-Smith *et al*., 1993; Middleton *et al*., 1993; Miller *et al*., 1995; Salzman, 1995; Szabó, 1995). The gastrointestinal epithelium represents an important interface between host and external environment, serving both as a surface for absorption and defense against ingested pathogens. In the oral cavity, a unique feature to be handled by the host defence is that the continuously replaced bacteria may obtain a firm anchorage on the nonshedding tooth surface and will thereby remain in close contact with the soft tissues surrounding the tooth for a long time and evoke inflammation. This chronic inflammatory disease of the soft and hard supporting tissues of the teeth is periodontitis, which is one of the most frequent human diseases. While periodontitis supports the protection against local microbial attack, this inflammatory reaction can also damage the surrounding cells and connective tissue structures, including alveolar bone causing tooth loss (see for review: Lindhe, 1995).

It has been well established that the inflammatory diseases of the periodontium are most frequently of bacterial origin. The

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Presented at 34th Meeting of the Continental European Division of the International Association of Dental Research, Madrid (Spain) 1997.

toxins, enzymes and metabolites of the bacteria (predominantly Gram-negative anaerobic) present in the dental plaque play a key role in the initiation of the inflammatory process, but the exact pathomechanism is far from being understood in detail (Slots, 1977; Stamm 1986; Listgarten, 1987; Lindhe, 1995). Although bacterial induction of iNOS has been demonstrated *in vitro* and has also been proposed to occur in the gastrointestinal epithelium *in vivo* (Teppermann *et al*., 1994; Eaves-Pyles *et al*., 1996), the role of iNOS in the pathogenesis of periodontitis has not been studied previously.

Recognizing the lack of information about the expression and role of iNOS and peroxynitrite in the development of periodontitis, in the present study we investigated whether iNOS is involved in the pathogenesis of experimentally-induced periodontitis. The presence of iNOS was detected by the measurement of iNOS activity and by localization of iNOS by immunocytochemistry. In addition, we have investigated the potential protective effect of mercaptoethylguanidine (MEG), a selective iNOS inhibitor (Southan *et al*., 1996) and peroxynitrite scavenger (Szabó *et al*., 1977), on the development of vascular leak of the gingivomucosal tissue and on alveolar bone loss associated with periodontitis. The data presented demonstrate the presence of iNOS in periodontitis and support the deleterious role of iNOS expression and peroxynitrite formation in the pathophysiology of this condition.

Methods

Experimental protocols

A total of 30 male Albino Wistar rats weighing from 280 to 400 g were used. Animals were lightly anaesthetized with surgical doses of doses of sodium pentobarbitone (35 mg kg^{-1}). Sterile, 2-0 black braided silk thread was placed around the cervix of the lower left first molar and knotted mesially according to Györfi *et al*. (1994). On the buccal, lingual and distal side of the tooth the tread was located subgingivally, while on the mesial side it was situated supragingivally. After the rats had recovered from the anaesthetic they were allowed to eat commercial laboratory food and drink tap water *ad libitum*. Animals were divided into 3 groups. In the first group ($n=16$) animals received i.p. vehicle (four times per day for eight days). From this group of animals, 7 rats were subsequently used for NOS determinations, 3 animals for immunohistochemistry and 6 animals for extravasation and bone resorption studies. In the second group ($n=7$), the rats were ligated as described above, received MEG treatment (30 mg kg^{-1}, i.p., four times per day for eight days), and were used for extravasation and bone resorption studies. Similar regimens of MEG treatment have previously been shown to exert anti-inflammatory effects, without causing notable side-effects (Southan *et al*., 1996; Brahn *et al*., 1997). In an additional (third) group of animals ($n=7$), the same procedure was performed as described above except ligation (sham operated control). These animals were used for the measurement of NOS activity.

Eight days after the operation, the animals were anaesthetized again (as above) and the mandibulae were excised, separated from the surrounding tissues and cut in half in a sagittal plane between the incisors. Approximately 3 mm thick gingivomucosal tissue stripe from the half of the lingual side of the second molar, circumferential (around) the mandibular first molar to the half of the buccal side of the second molar was excised on both sides from animals for iNOS activity assay, iNOS immunohistochemistry and extravasation measurement. For iNOS activity assay the gingivomucosal tissues were quick frozen in liquid nitrogen, stored at $-80^{\circ}C$ before the measurements. The gingivomucosal samples of all rats throughout the experiments were removed by the same investigator. The half mandibles were processed for alveolar bone loss measurement. The animal protocol was approved by the Institutional Ethics Committee.

Nitric oxide synthase assay

Calcium-dependent conversion of L-arginine to L-citrulline in homogenates of gingivomucosal tissues served as an indicator of tissue cNOS activity, and calcium-independent conversion of L-arginine to L-citrulline in homogenates of gingivomucosal tissues served as an indicator of tissue iNOS activity (Szabó *et al*., 1994). Gingivomucosal tissues were taken from the animals of group one and three and were homogenized on ice, in a buffer composed (in mM) Tris.HCl 50, EDTA 0.1 and phenylmethylsulphonyl fluoride 1 (pH 7.4), with a Tissue Tearor 985-370 homogenizer (Biospec Products, Racine, WI, U.S.A.). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described by Szabó *et al*. (1994). Briefly, for the measurement of cNOS activity, homogenates (30 μl) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 20 min at 22°C. For the measurement of iNOS activity, homogenates (30 μl) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and EGTA (2 mM) for 20 min at 22°C. Reactions were stopped by dilution with 0.5 ml of ice-cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). In parallel experiments, all NOS activity was inhibited by the co-incubation of the samples with the non-isoenzyme-specific NOS inhibitor N^G-methyl-L-arginine (L-NMA, 1 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured by a Wallac scintillation counter (Wallac, Gaithersburg, MD, U.S.A.). Due to the small sample size, NOS activity was determined in pooled gingivomucosal samples obtained from 7 animals in the first and third experimental group, and determinations were performed in quadruplicate. The mean and s.e.mean of these quadruplicate determinations are presented in Figure 1. Results similar to the ones presented were obtained in 3 different experimental days, as measured in 3 different sets of pooled samples.

Immunohistochemistry

Three ligated rats from group one were terminally anaesthetized with intraperitoneal injections of sodium pentobarbitone and were perfused with phosphate-buffered saline (PBS) followed by a fixative containing 3% paraformadehyde, 0.1% glutaraldehyde and 150 ml saturated picric acid (pH 7.3). Small pieces of gingivomucosal tissues encircling the mandibular first molars were dissected out on both sides, as described in the experimental protoxol, and immersed in a same, but glutaraldehyde-free fixative overnight. Next, 30–40 μm thick sections were cut on a Vibratome and rinsed in PBS. The sections were incubated with primary rabbit polyclonal antibodies generated to peptides from mouse iNOS (a kind gift of Dr M. Currie, Monsanto Co., St. Louis, MO, U.S.A.). Tissues were incubated with the antibody at a dilution of 1:1000 in PBS for 48 h at 4°C. Following rinses in PBS, the sections were reacted with a biotinylated goat-anti rabbit IgG

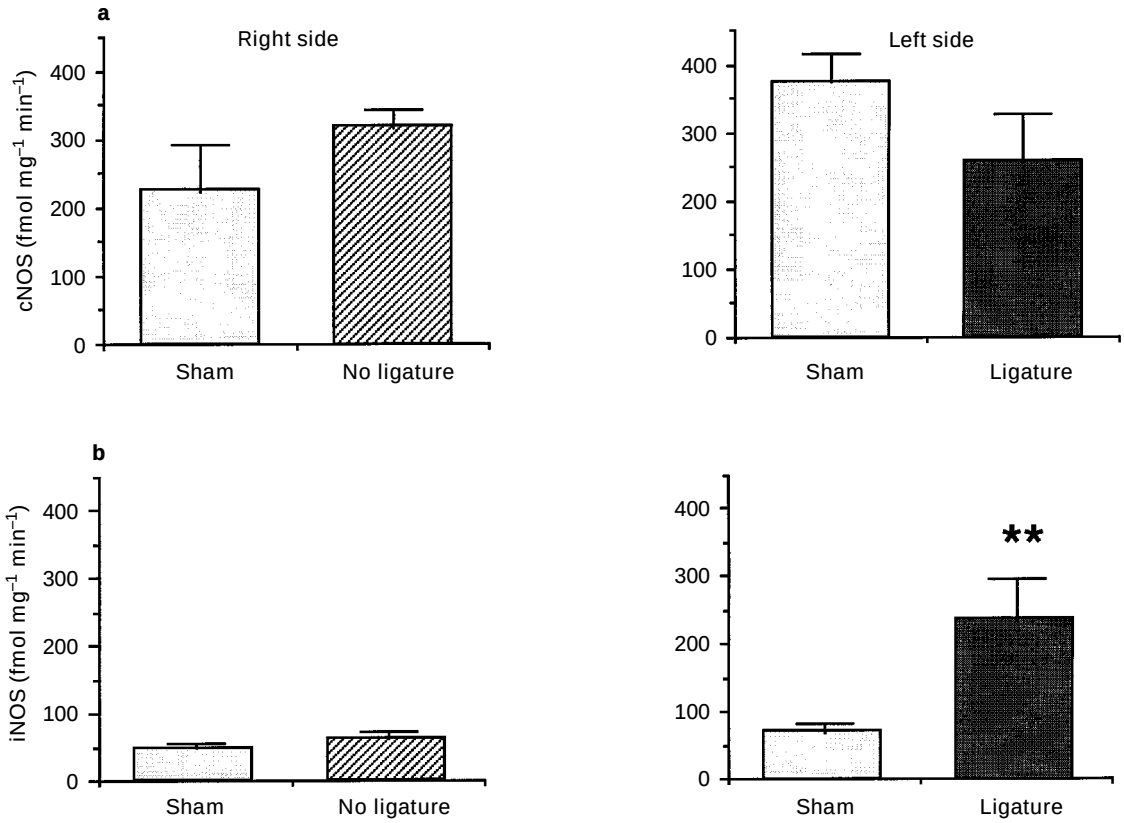

Figure 1 The effect of ligation ($n=7$) and sham operation ($n=7$) on (a) cNOS and (b) iNOS activity of the gingivomucosal tissue around left and right first molar. Results of a typical experiment from pooled samples are shown (mean ± s.e.mean of quadruplicate determinations). $^{**}P<0.01$ sham operated vs ligated rats.

(1:500 dilution), then with an avidin-biotin-peroxidase complex (1:250, both of Vectastatin ABC Kits, Vector Laboratories) for 1 h at room temperature. Between each incubation step, sections were washed three times in 0.1 M PBS. All incubations were performed in 0.1 M PBS containing 1% normal goat serum and 0.1% sodium azide. After the final wash, tissues were further rinsed in 50 mM Tris-HCl buffer (pH 7.6), and reacted in Tris-HCl buffer containing 0.05% 3,3-diaminobenzidine (DAB) and 0.002% H_2O_2 to visualize the antigens. After the DAB reaction, the sections were mounted on gelatin-coated slices, air dried and coverslipped with DePeX. As a control for immunohistochemical specificity the antisera were omitted before the immunohistochemical staining.

Measurement of vascular permeability by Evans blue extravasation

In a separate set of experiments in animals from the first group ($n=6$) and of the second group ($n=7$) periodontitis was induced and in the second group MEG treatment performed as above. Eight days after anaesthesia (see above), the trachea was catheterized to ensure spontaneous respiration. Cannulae were inserted into the right femoral vein and artery. After surgery, the animals were allowed to stabilize for a 30 min period. Systemic blood pressure was monitored through the femoral artery catheter by a manometer. Constant body temperature of the animals (38°C rectal temperature) were maintained, applying periodical heating. To assess vascular permeability, animals received Evans blue (2.5%; Reanal, Hungary, dissolved in physiological saline, at a dose of 50 mg kg^{-1}) via the femoral venous catheter. Five min later a cannula was introduced into the abdominal aorta. Ten minutes later the right atria was cut and the dye remaining in the gingivomucosal capillaries was removed by retrograde intraaortic injection of 40 ml isotonic saline solution. Extravasated Evans blue in the excised gingivomucosal tissue samples was extracted with 1 ml formamide for 48 h at room temperature for spectrophotometric determination at 620 nm and expressed as μg g^{-1} gingivomucosal tissue (Gamse *et al*., 1980; Györfi *et al*., 1994).

Measurement of alveolar bone loss

In the same set of experiments that was used for the plasma extravasation measurements, the distance from the cemento-enamel junction of first lower molars to the alveolar crest was measured with a modification of the method of Crawford *et al*. (1978), by use of a preparation microscope (Wild M 32) equipped with a videocamera (Philips) and a TV monitor, and a custom-made analyzer system, which displayed the distance digitally. Calibrations were made with commercial standards. The magnification at the screen was 69 fold, the sensitivity of the measurement was approximately 20 μm. Recordings were made along the median axis of the lingual surface of the mesial and mediolingual roots of the lower first left and right molars. These measurements were performed by an independent investigator who was unaware of the treatment regimens. The alveolar bone loss induced by the ligature was expressed as a difference between the left and the right side.

Materials

Sodium pentobarbitone (Nembutal) was obtained from Phylaxia-Sanofi, Budapest, Hungary); 2-0 black braided silk was purchased from Medicor (Budapest, Hungary). Mercap-

toethylguanidine (MEG) was prepared as previously described (Southan *et al*., 1996). All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Statistical analysis

All values in the figures and in the text are expressed as mean ± s.e.mean of n observations. Student's unpaired t test was used to compare means between groups. A P value of less than 0.05 was considered to be statistically significant.

Results

Increased iNOS activity in periodontitis

There were significant amounts of cNOS activity in the gingivomucosal tissues of control (sham-ligated) rats, and there was also a small amount of iNOS activity detectable in the gingivomucosal tissues of these animals (Figure 1). Ligation caused a significant, more than 3 fold, increase in the iNOS activity, whereas did not significantly affect iNOS activity on the contralateral side (Figure 1). Ligation, on the ipsilateral, but not on the contralateral side, also tended to cause an approximately 15% decrease in the cNOS activity (Figure 1).

Localization of iNOS in periodontitis

NO significant iNOS staining was observed in control samples, except a few immunoreactive connective tissue cells in the tunica propria (Figure 2a). Eight days following ligation, there was a marked increase in the number of iNOS-immunoreactive inflammatory cells in the tunica propria. This iNOS immunoreactivity was mainly localized to macrophages, mast cells, plasma cells, lymphocytes and PMNs (Figure 2b). Some blood vessels and a few fibroblasts also showed a slight immunoreactivity (Figure 2c). The basal layer and the uppermost layer of the epithelial lining of the gingiva showed heavy immunostaining for iNOS, otherwise the epithelium was moderately reactive (Figure 2d). The epithelial surface was keratinized (Figure 2d). It is noteworthy that a few immunoreactive nerve bundles were also seen in the connective tissue in control as well as in the ligated side (Figure 2c).

Effect of MEG on plasma extravasation and bone destruction

Before the measurement of Evans blue extravasation, mean arterial pressure of vehicle-treated and MEG-treated animals was recorded. In agreement with previous studies (Southan *et al*., 1996), MEG treatment did not affect mean arterial blood pressure, indicative of a lack of effect on constitutive, endothelial NO production (vehicle-treated: 108 ± 12 mmHg; $n=6$ and MEG-treated: 104 ± 9 mmHg; $n=7$).

After Evans blue injection, in contrast to the other part of the mouth, in the gingiva around the neck of the teeth a definite blue belt could be observed even in non-ligated control animals (not shown). Ligation significantly increased Evans blue extravasation in gingivomucosal tissue compared to the contralateral side (79.3 ± 12.8 μg g^{-1} vs 34.5 ± 6.1 μg g^{-1}, $n=6$, $P<0.05$). MEG treatment prevented this increase in Evans blue extravasation, but did not change the Evans blue content of the contralateral side (52.4 ± 6.9 μg g^{-1} vs 45.2 ± 8.1 μg g^{-1}, $n=7$, respectively) (Figure 3). The difference in the increase in Evans blue content between the ligated and contralateral side was 44.7 ± 12.2 μg g^{-1} in the control group vs 7.1 ± 10.7 μg g^{-1} ($P<0.05$) in the MEG-treated group.

The alveolar bone loss between the lower first left and the right first molars induced by the left side ligature were at mesial root 0.24 ± 0.05 mm, at mediolingual root 0.26 ± 0.04 mm. MEG treatment resulted in a significant inhibition of alveolar bone loss after ligation. In the MEG-treated animals, the bone loss at the mesial root was 0.08 ± 0.02 mm ($P<0.01$) (Figure 4), and at the mediolingual root 0.09 ± 0.03 mm ($P<0.01$) (Figure 5).

Discussion

iNOS expression and increased activity in periodontitis: comparison to the effect of endotoxin challenge

In the present study, a well established rat model of periodontitis was utilized, which involves a ligature around the cervix of the mandibular first molar tooth. A similar model has previously been used in several species (Rovin *et al*., 1966; Schroeder & Lindhe, 1975; Meitner, 1975; Sallay *et al*., 1984). In this model, ligation acts as (i) a mechanical trauma on the dentogingival area, thereby reducing tissue integrity and allowing for intense host-plaque interaction and (ii) a plaque-formation-promoting factor, thus increasing the number of bacteria. Initiation of periodontal disease by bacteria is well-documented, and the end result, destruction of the alveolar bone and other connective tissues is readily observed. However, the molecular events that promote these alterations are incompletely understood.

The present results clearly demonstrated that iNOS is expressed in periodontitis, with localization mainly in inflammatory and epithelial cells. In order to determine the extent or importance of iNOS induction after ligature, we compared the iNOS activity observed after 8 days of ligature with the iNOS activity seen after i.p. administration of *E. coli* lipopolysaccharide (LPS) (15 mg kg^{-1}) for 6 h. Systemic administration of bacterial LPS triggers the expression of iNOS in many tissues and 6 h is the peak for iNOS expression in this model (Szabó *et al*., 1994). Indeed, there was a significant increase in iNOS activity in response to systemic administration of LPS (from 59 ± 6 fmol mg^{-1} min^{-1}) to 107 ± 35 fmol mg^{-1} min^{-1} ($n=3$, $P<0.01$). However, it appears that the induction of iNOS in response to ligation induced a more significant expression of iNOS (see Figure 1) when compared to systemic administration of LPS.

LPS of the dominant Gram-negative bacteria in periodontitis (Slots, 1977; Sallay *et al*., 1984; Page, 1991; Lindhe, 1995) may directly trigger resident or immigrant cell populations for expression of iNOS or provoke an immune response that results in the release of cytokines that subsequently may induce iNOS. Proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), are present in cervicular fluid from inflamed sites at significant levels (see for review: Page, 1991; Birkedal-Hansen, 1993; Seymour *et al*., 1993) and may be involved in the process of iNOS expression. These cytokines are known to play a key role in the expression of iNOS in response to systemic administration of LPS (Szabó, 1995).

Large NO concentrations produced by iNOS have a role in nonspecific immunity, cytostatic/cytotoxic against invading microbial pathogens (Mancinelli & McKay, 1983; Hibbs *et al*., 1990; Wong & Billiar, 1995). However, NO or peroxynitrite may also damage the host tissues via multiple mechanisms

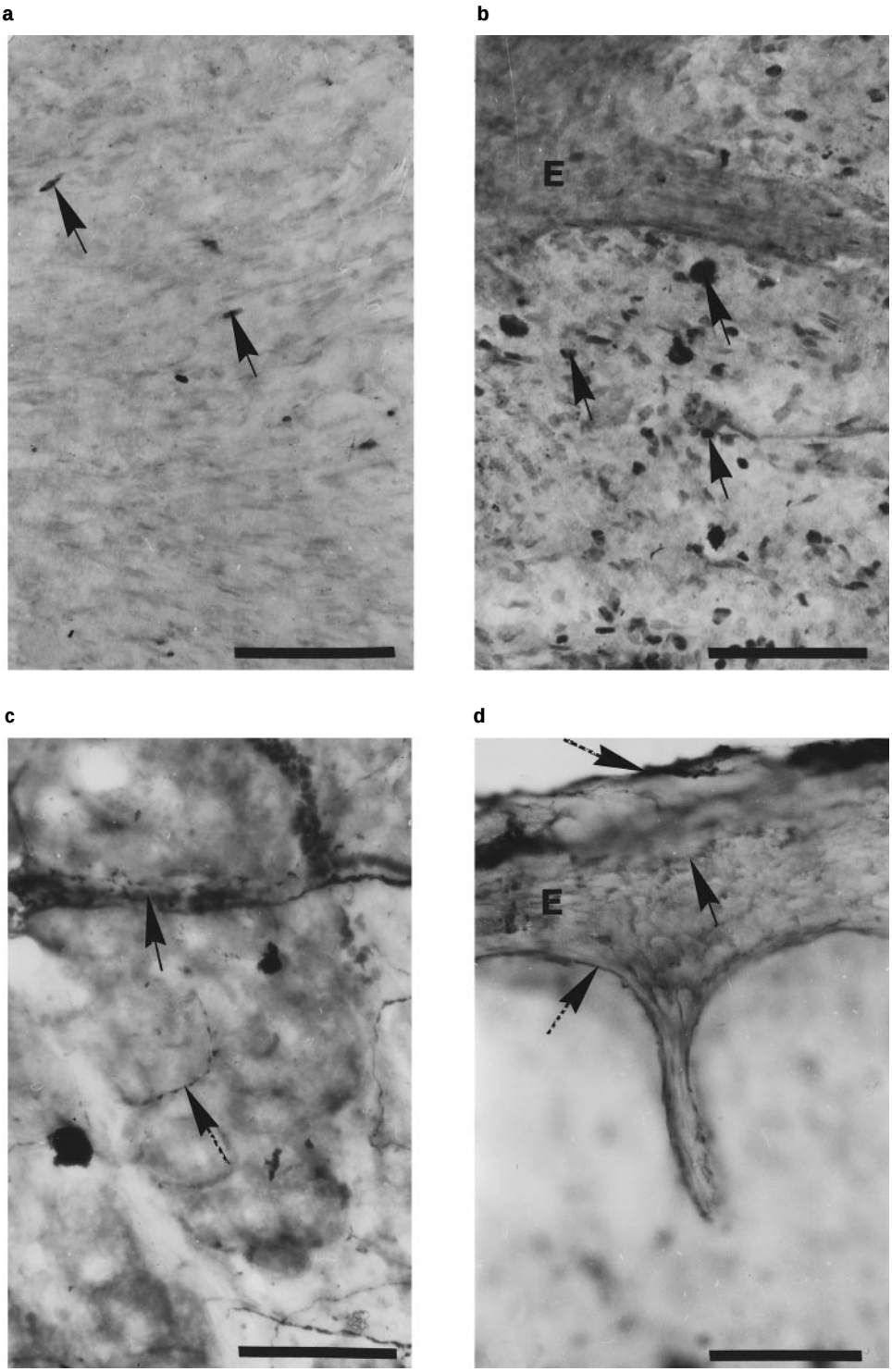

Figure 2 Photomicrographs of iNOS immunoreactive elements in rat gingivomucosal tissue. Bar scale = 100 μm. (a) A part of the gingiva from the control animal. Arrows point the moderately immunoreactive connective tissue cells. (b) A part of the gingiva from ligated rats. Arrows show the immunoreactive cells in the connective tissue (macrophage, plasma cell, lymphocyte). (c) A part of the tunica propria from ligated rats with a moderately immunoreactive blood vessel (arrow). Dotted arrow shows a positive nerve bundle. (d) A part of the gingiva from ligated rats. Arrow shows the moderate immunoreactive epithelial lining (E). Dotted arrows point the basal and uppermost layers of epithelium showing heavy immunoreactivity.

(Hibbs *et al*., 1990; Beckman *et al*., 1990; Beckman & Koppenol, 1996; Szabó, 1996; Kröncke *et al*., 1997). It is conceivable that wall components of Gram-negative bacteria killed by NO or peroxynitrite trigger a positive feedback cycle, whereby bacterial wall products may induce more NO production. Although the production of NO or peroxynitrite serves to induce killing or stasis of the invading microorganisms, the overproduction of these species may also lead to cytotoxicity towards the host tissues. It is noteworthy that several of the enzymatic pathways for degradation of the gingival host structures may be regulated in a fashion similar to iNOS. Plasminogen activators and most matrix metalloproteinases are under growth factor/cytokine transcriptional control (levels are low or non-detectable in health and are elevated markedly along the continuum of increasing disease severity) and are down-regulated by glucocorticoids (see for

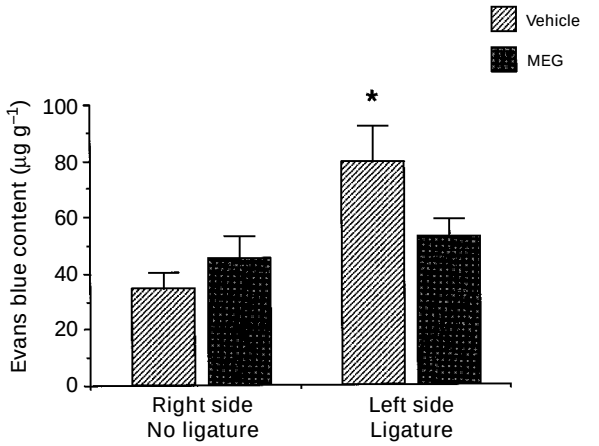

Figure 3 The effect of ligature on Evans blue content of the gingivomucosal tissue in vehicle-treated animals ($n=6$) and MEG-treated animals ($n=7$). *$P<0.05$ in control left vs right side. Data are presented as means ± s.e.mean.

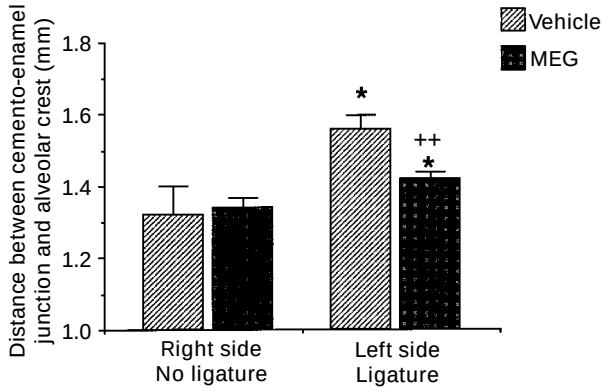

Figure 4 The distance between cemento-enamel junction and alveolar crest at mesial root of the first molar in vehicle-treated animals ($n=6$) and MEG-treated animals ($n=7$). *$P<0.05$ left vs right side, ++$P<0.01$ control left vs MEG left side. Data are presented as mean ± s.e.mean.

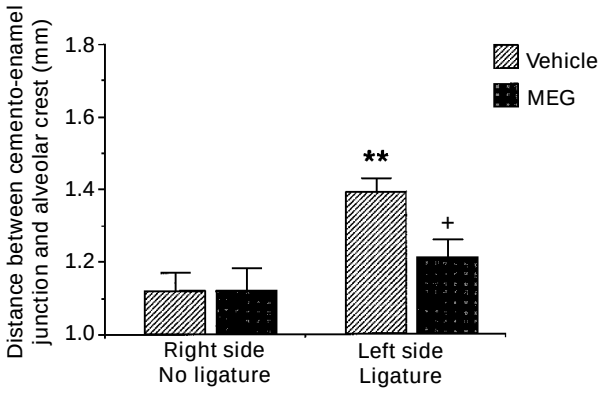

Figure 5 The distance between cemento-enamel junction and alveolar crest at mediolingual root of the first molar in vehicle-treated animals ($n=6$) and MEG-treated animals ($n=7$). **$P<0.01$ left vs right side, +$P<0.05$ control left vs MEG left side. Data are presented as mean ± s.e.mean.

review: Birkedal-Hansen, 1993; Page, 1991). Dysregulated production of cytokines and other proinflammatory mediators may lead to an excessive local amplification of the immune response, which may be in part responsible for periodontal tissue destruction during the progression of periodontal disease.

It is important to know the cellular distribution of iNOS, in order to improve our understanding of the pathomechanism of periodontitis. Similar to the previous observations (Sallay *et al*., 1982; Györfi *et al*., 1994), 8 days ligature around the mandibular 1st molar of the rat resulted in accumulation of inflammatory cells in the connective tissue adjoining the ligature. These inflammatory cells were heavily immunoreactive to iNOS, together with basal and the uppermost layer of the epithelial lining of the gingiva. However, in our experiment iNOS immunoreactivity was moderate or not detected in vascular structures or in fibroblasts. Although staining was only sparsely seen in these elements, levels of iNOS that are below the immunocytochemistry detection limit cannot be excluded.

Role of iNOS in periodontitis: effects of MEG treatment

In periodontitis, iNOS expression may have beneficial as well as detrimental roles. Beneficial effects may include antimicrobial activity, immune modulation, inhibition of microvascular thrombosis, as well as increased tissue perfusion. On the other hand, detrimental effects may include a cytotoxic action towards the host tissues including alveolar bone. Development of chronic destructive inflammation of the periodontium may be a maladaptive process. The effects of an excess of NO in the gingivomucosal tissue may contribute to the development of the frequently found clinical symptoms of periodontitis in man. The gingival redness may be explained by a vasodilator effect of NO, and the gingival swelling may be explained by the vascular permeability-increasing effect of NO. The increased tendency of the soft tissue to bleed on gentle probing may be due to the inhibitory effect of NO on platelet aggregation and adhesion. Lastly, the increased alveolar bone resorption may be due to the stimulating effect of NO on the activity of the osteoclasts (see for reviews: Moncada, 1992; Hukkanen *et al*., 1995).

To investigate the potential role of iNOS in the pathophysiology of periodontitis, we used mercaptoethylguanidine, a novel inhibitor of NOS, with selectivity towards the inducible isoform (Southan *et al*., 1996) and which has additional, peroxynitrite scavenging properties (Szabó *et al*., 1997). MEG, at doses similar to the ones used in the present study, has previously been shown to ameliorate the cardiovascular failure and the development of arthritis in the rat (Southan *et al*., 1996; Zingarelli *et al*., 1997a; Brahn *et al*., 1997). Our results demonstrated that MEG exerted a marked inhibitory effect on the plasma extravasation and reduced the degree of bone resorption during periodontitis.

Our study also confirmed earlier findings, that one of the characteristic signs of inflammation, Evans blue extravasation, was higher on the ligated side on the 8th days, than on the opposite side (Györfi *et al*., 1994), and that various NOS inhibitors (aminoguanidine, N^G-methyl-L-arginine) reduced the subsequent endotoxin-induced vascular leakage in intestine at the time of the iNOS expression (László *et al*., 1995).

There may also be a small degree of subclinical spontaneous baseline peridontal inflammation in the rat (Kindlova & Scheinin, 1968), as indicated by detectable levels of 'basal' extravasation. This phenomenon may be related to the effects of the normal oral flora. Inhibition of this 'baseline' iNOS, together with basal gingivomucosal NO production by cNOS (Kerezoudis *et al*., 1993a; Lohinai *et al*., 1997) may explain the decreased resting vascular extravasation caused by L-NAME, a non-isoform-selective inhibitor of NO synthases (Benedek *et al*., 1996). In certain areas of the upper airways iNOS also seems to be

constitutively expressed in the epithelial cells (Lundberg, 1996), probably because the infectious agents are present constitutively. In the rat gingiva the basal cell layer of the epithelium (Kerezoudis *et al*., 1993b) and in the cat gingiva throughout the epithelium (Lohinai *et al*., 1997), nicotinamide adenine dinucleotide phosphate diaphorase activity (NADPH-d, one possible histochemical marker for NOS) was also localized.

Our experiment confirmed that ligature induces significant alveolar bone resorption (Kennedy & Polson, 1973; Zappa *et al*., 1991), as measured at 8 days, an effect which was blocked by MEG treatment. In a rat experimental arthritis model, a NOS inhibitor (N^G-methyl-L-arginine) also suppressed pathological changes like cartilage breakdown and osteomyelitis (Stefanovic-Racic *et al*., 1994). IL-1 and TNF-α are potent inducers of bone resorption *in vitro* (Gowen & Mundy, 1986; Bertolini *et al*., 1986) and in rats receiving IL-1 beta with ligatures, an extensive bone resorption had occurred (Koide *et al*., 1995). Moreover, iNOS has also been demonstrated in osteoblasts and osteoclasts, and the NO produced by iNOS in these cells have been implicated in inflammatory bone resorption (Hukkanen *et al*., 1995; Sunyer *et al*., 1996). The NADPH-d positive cells on the alveolar bone surface of the periodontal ligament and in alveolar bone marrow crypts may be involved in the NO-mediated alveolar bone resorption (Kerezoudis *et al*., 1993b). The exact mechanism by which MEG inhibits alveolar bone resorption remains to be established in further studies.

Mechanisms of action of MEG

iNOS-derived NO, peroxynitrite, oxyradicals and products of the inducible isoform of cyclo-oxygenase (COX) have independently been proposed as important factors in the pathogenesis of various forms of inflammation (see: Pfeilschifter *et al*., 1996; Pairet & Engelhardt, 1996; Vane & Botting, 1996; Szabó, 1996). MEG is a member of the mercaptoalkylguanidines, a class of NOS inhibitors with selectivity towards the inducible isoform (Southan *et al*., 1996). MEG, as opposed to non-iNOS selective inhibitors of NOS, does not inhibit the catalytic activity of constitutive endothelial NOS in therapeutically relevant doses, and does not raise blood pressure in anaesthetized rats in the doses used (Southan *et al*., 1996), as confirmed in the present study. The lack of inhibition of ecNOS activity by MEG is a distinct advantage of this agent, when compared to inhibitors with limited or so-called isoform-selectivity (e.g. the prototypical NOS inhibitor N^G-methyl-L-arginine) (see Introduction). Recent work with MEG has revealed that this agent, in addition to its inhibitory effect on iNOS, also exhibits a modest inhibitory effect on the constitutive and inducible isoforms of COX (Zingarelli *et al*., 1997b). Moreover, MEG, as a thiol, is a scavenger of oxyradicals and peroxynitrite (Shapira *et al*., 1957; Kozak & Arient, 1973; Szabó *et al*., 1997). The multiple anti-inflammatory effects of MEG, although evidently advantageous from a practical and a potential therapeutic point of view, make it very difficult to dissect the *in vivo* mode of action of this agent.

In conclusion, in the present study we presented evidence for an increased iNOS production by resident and infiltrating inflammatory cells in the pathogenesis of plaque-associated periodontal disease. The expression of iNOS may the consequence of direct (bacterial cell wall products) and/or an indirect (pro-inflammatory cytokines) immunostimulation. The excessive local production of NO, on the one hand, may beneficial by eliminating invading bacteria, and, on the other hand, may be detrimental by inducing inflammation and increased bone resorption. We propose that inhibition of iNOS in periodontitis reduces the degree of inflammation and is beneficial, at least in the short-term.

The authors express their gratitude to Ágnes Gara, Dr György Haskó, Dr György Nádasy, Ferencné Adamkó and Bodó Márta for excellent technical help and/or assistance. This work was supported by Research Grants of the Hungarian National Research Funds OTKA No. F-20469, 017414, ETK I13 and ETT 02227, and by the Blue & Blue BT (Budapest, Hungary).

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(Received May 21, 1997
Revised September 29, 1997
Accepted October 13, 1997)