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Pyrrolidine dithiocarbamate reduced experimental periodontitis

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Abstract

Nuclear factor-κB (NF-κB) is a transcription factor which plays a pivotal role in the induction of genes involved in physiological processes as well as in the response to injury and inflammation. Dithiocarbamates are antioxidants which are potent inhibitors of NF-κB. We postulated that pyrrolidine dithiocarbamate (PDTC) would attenuate inflammation. In the present study, we have investigated the effects of PDTC, in a rat model of periodontitis. Periodontitis was induced in rats by placing around the lower left first molar a 2/0 braided silk. At day eight the gingivomucosal tissue encircling the mandibular first molar was removed for biochemical and histological analysis. At day eight ligations significantly induced an increase neutrophil infiltration as well as the gingivomucosal tissue expression of TNF-α and iNOS as well as nitrotyrosine formation and poly (ADP-ribose) polymerase activation. Ligation significantly increased Evans blue extravasation in gingivomucosal tissue and alveolar bone destruction. Intraperitonial injection of PDTC (10 mg/kg daily for eight days) significantly reduced all of the parameters of inflammation as described above. These data demonstrate that PDTC exerts an anti-inflammatory role during experimental periodontitis and is able to ameliorate the tissue damage associated with ligature-induced periodontitis.

Keywords: Pyrrolidine dithiocarbamate; Nuclear factor-κB; Alveolar bone loss; Reactive oxygen specie; Periodontal disease

1. Introduction

The chronic inflammatory disease of periodontal tissues is periodontitis, one of the most frequent human diseases (Loos, 2005; Loos et al., 2005). While periodontitis supports the protection against local microbial attack, this inflammatory reaction may also damage the surrounding cells and connective tissue structures, including alveolar bone causing tooth loss (Han et al., 2005; Lindhe and Nyman, 1987).

It has been recently demonstrated that the most frequent cause of periodontitis are bacteria. The toxins, enzymes and metabolites of the bacteria present in the dental plaque play a key role in the initiation of the inflammatory process (Listgarten, 1987). The more current terminology aggressive periodontitis is an inflammatory disease with severe periodontal destruction, occurring in the early twenties, teens or before. Some reports have evaluated polymorphonuclear cells functions in patients affected by early-onset periodontitis, but the results are discordant. Some authors reported a defective chemotactic response to formyl-met-leu-phe (Suzuki et al., 1984) and to complement-derived C5a (Genco et al., 1986). Contrary to the above mentioned studies, chemotaxis of early-onset periodontitis polymorphonuclear cells has been reported to be normal or increased (Repo et al., 1990). Moreover, recent studies have clearly demonstrated that inducible nitric oxide synthetase (iNOS) and reactive oxygen species (ROS) plays an important role in the inflammatory process as well as in the bone resorption (Di Paola et al., 2004).

One of the major signal-transduction pathways that is activated in response to oxidant stress is that of the nuclear transcription factor nuclear factor κB (NF-κB), which is crucial for cell

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survival, cell proliferation and immune responses via expression of its target genes (Baeuerle and Henkel, 1994; Chen et al., 1999). In quiescent cells, the active form of NF-κB in cytosol remains bound to the inhibitory molecule, IκBα (inhibitor of NF-κB) (Chen et al., 1999). Upon stimulation by pro-inflammatory cytokines or oxidants, IκBα is phosphorylated by the upstream kinase, I kB kinase (IKK), which leads to the polyubiquitination and degradation of IkB by proteases, causing the release and translocation of NF-κB complex into the nucleus (Chen et al., 1999). Activated NF-κB subsequently binds to specific DNA sequences and regulates the expression of its target genes, which mediate inflammatory response, apoptosis and carcinogenesis. Recently it has been demonstrated that porphyromonas gingivalis, a major etiological pathogen of adult periodontitis characterized by alveolar bone resorption, induced NF-κB activation, and the induction of osteoprotegerin in human microvascular endothelial cells by the pathogen was blocked by the inhibitors of NF-κB (Kobayashi-Sakamoto et al., 2004).

The dithiocarbamates represent a class of antioxidants reported to be potent inhibitors of NF-κB in vitro (Schwartz et al., 1996; Somers et al., 2000). The metal-chelating properties of the diethyl derivative of dithiocarbamate (diethyldithiocarbamate, DDTC) have been exploited for decades for the treatment of metal poisoning in humans (Reisinger et al., 1990). More recently, DDTC has been used to retard the onset of acquired immune deficiency syndrome (AIDS) in human immunodeficiency virus (HIV)-infected individuals (Schreck et al., 1991), a phenomenon thought to be related to its effect on NF-κB activation(Schreck et al., 1991; Topping and Jones, 1988). In this regard, the most effective NF-κB inhibitor appears to be the pyrrolidine derivative of dithiocarbamate (pyrrolidine dithiocarbamate, PDTC) as a result of its ability to traverse the cell membrane and its prolonged stability in solution at physiological pH (Topping and Jones, 1988). Moreover, we have previously demonstrated that PDTC exerts a therapeutic benefit in acute and chronic inflammatory experimental conditions in which activation of NK-κB plays a major role (Cuzzocrea et al., 2002).

Base on this previous observation, the present study was designed to evaluate the effects of PDTC in animal models of periodontitis. In particular, we investigate the effects of PDTC on (1) mean arterial blood pressure (2) inducible nitric oxide synthase expression (3) polymorphonuclear infiltration (myeloperoxidase activity) (4) nitration of tyrosine residues (an indicator of the formation of peroxynitrite by immunohistochemistry), (5) poly (ADP-ribose) polymerase activation (6) TNF-α production (7) measurement of vascular permeability (8) alveolar bone loss (radiography) and tissue damage (histology).

2. Materials and methods

2.1. Surgical procedure

Male Sprague–Dawley rats (280–400 g) were lightly anaesthetized with surgical doses of sodium pentobarbitone (35 mg/kg). Sterile, 2-0 black braided silk thread was placed around the cervix of the lower left first molar and knotted medially as previously described (Di Paola et al., 2004). After the rats had recovered from the anaesthetic they were allowed to eat commercial laboratory food and drink tap water ad libitum. Animals and the study protocol were approved by the Institutional Animal Care and User Committee of the University of Messina.

2.2. Measurement of arterial blood pressure indirectly in conscious rat

Mean arterial blood pressure in conscious rats was measured by a Blood Pressure Recorder (UGO BASILE, Biological Research Apparatus, 21025 Comerio, Italy), After a week period, rats were treated as described below and blood pressure was measured 30 min before and after each i.p. injection, on each of the 8 days of treatment. To measure arterial blood pressure, rats were housed for 30 min in a warmed room (28–30 °C) and then a tail cuff, consistently about 2 cm from the base of the tail was placed and arterial blood pressure was measured. Heart rate was detected by a pulse rate counter placed after the tail cuff.

2.3. Experimental groups

Rats were randomly allocated into the following groups: *Ligature+vehicle group*: rats were subjected to ligature-induced periodontitis and animals received vehicle intraperitoneally (i.p.; daily treatment for eight days). *Ligatures+PDTC group*: rats were subjected to ligature-induced periodontitis and animals received PDTC (10 mg/kg i.p., daily for eight days). At 8 days after the ligature-induction of periodontitis the rats ($N=30$ from each group) were sacrificed in order to evaluate the various parameters ($N=10$ from each parameter) as described below. ($N=10$ from each group for each parameter) were sacrificed. The dose of PDTC used in the present studies was taken from previous studies showing dose efficacy without side effect (for 10 days treatment) in models of chronic inflammation (Cuzzocrea et al., 2002).

2.4. Measurement of vascular permeability by Evans blue extravasations

Vascular permeability was determinate as previously described (Gyorfi et al., 1994). Briefly, animals received Evans blue (2.5% dissolved in physiological saline, at a dose of 50 mg/kg) via a femoral venous catheter. 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 gingivomucosal tissue (Gyorfi et al., 1994).

2.5. Measurement of alveolar bone loss

The distance from the cementoenamel junction of first lower molars to the alveolar crest was measured with a modification of the method of Crawford et al. (1978). 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 as previously described (Di Paola et al., 2004). 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.

2.6. Histological examination

For histopathological examination, biopsies of gingival and mucosal tissue from the buccal and lingual aspect of the teeth were taken 8 days after the ligature-induction of periodontitis. The tissue slices were fixed in 10% neutral-buffered formaldehyde for 5 days, embedded in paraffin, and sectioned. The sections, orientated longitudinally from the teeth crowns, were stained with trichrome stain. The total number of infiltrating leukocytes (e.g., neutrophils and mononuclear cells) in cortical interstitial spaces from gingival and mucosa tissues were assessed quantitatively by counting the number of polymorphonuclear cells in 20 high power fields.

2.7. Radiography

Mandibles were placed on a radiographic cassette (ADCC HR cassette Agfa, Belgium) at a distance of 95 cm from the X-ray source. Radiographic analysis of no-ligated and ligated mandibles was performed, using computed radiography (CR) system, by X-ray machine (Philips X12 Germany) with a 40 kV exposure for 0.025 s (2.5 mAs), and high-resolution photostimulable phosphor imaging plate (ADC MD40 code 15 Imaging Plate Agfa, readout with ADC Solo Agfa Image reader, Belgium). The radiographic analysis of at eight day after ligature placement revealed bone matrix resorption in the lower first left after ligation as previously described (Di Paola et al., 2004).

2.8. Myeloperoxidase activity

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined in gingivomucosal tissue, as previously described (Mullane et al., 1985). Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol/min of peroxide at 37 °C and was expressed in milliunits/g of wet tissue.

2.9. Immunohistochemical localization of TNF-α, iNOS, nitrotyrosine and poly (ADP-ribose) polymerase

After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) H_2O_2 in 60% (v/v) methanol for 30 min. The sections were then incubated overnight with primary anti-TNF-α (1:500 dilution) antibody, anti-nitrotyrosine antibody (1:1000 dilution), primary anti-PAR (1:500 dilution), primary anti-iNOS (1:500 dilution) with control solutions including buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (DBA, Milan, Italy). Immunocytochemistry photographs ($N=5$ photos from each samples collected from all rats in each experimental group) were assessed as previously described (Di Paola et al., 2004) by densitometry analysis by using Optilab Graftek software on a Macintosh personal computer.

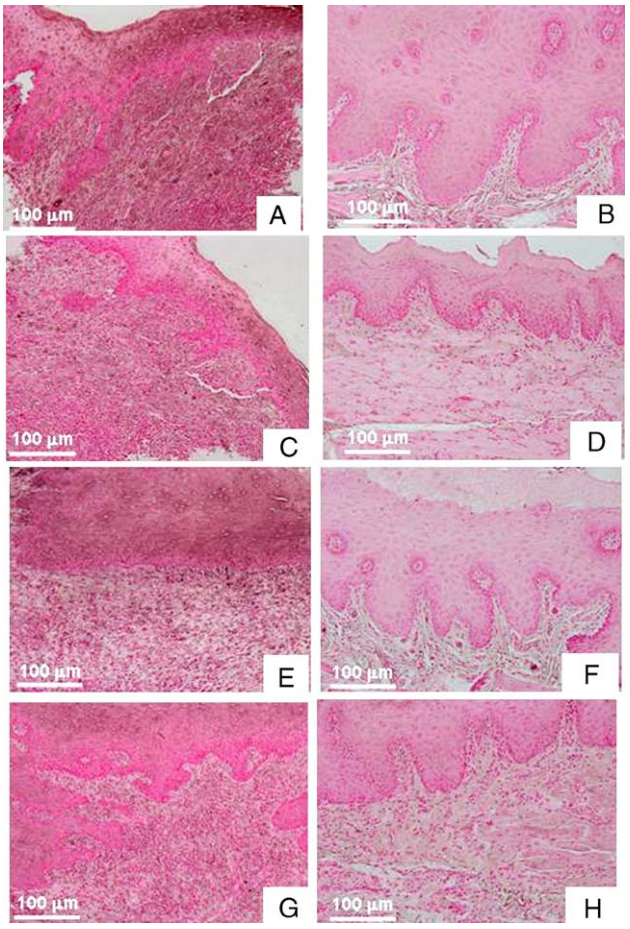

Fig. 1. Immunohistochemical staining for TNF-α, iNOS, nitrotyrosine and poly (ADP-ribose) polymerase formation. Positive staining for TNF-α (A), iNOS (C), nitrotyrosine (E) and poly (ADP-ribose) polymerase (G) was observed in gingivomucosal tissue after ligature. In gingivomucosal tissue of PDTC (10 mg/kg i.p., daily for eight days) treated rats no positive staining was observed for TNF-α (B), iNOS (D), nitrotyrosine (F) and poly (ADP-ribose) polymerase (H). Figure is representative of at least 3 experiments performed on different experimental days.

2.10. Materials

Primary anti-nitrotyrosine antibody was obtained from Upstate Biotech (DBA, Milan, Italy). All other reagents and compounds used, were obtained from Sigma Chemical Company (Sigma, Milan, Italy).

2.10.1. Data analysis

All values in the figures and text are expressed as mean± standard error of the mean of N observations, where N represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissues section collected from all the animals in each group. The results were analyzed by Student's

unpaired *t*-test. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. Effects of PDTC on TNF-α and iNOS expression, nitrotyrosine formation and poly (ADP-ribose) polymerase activation in periodontitis

Sections of gingivomucosal tissues from the contralateral side did not reveal any immunoreactivity for TNF-α, iNOS, nitrotyrosine or for anti-poly (ADP-ribose) polymerase (data not shown) within the normal architecture. At eight days following ligation, positive staining for TNF-α (Figs. 1A and 2A), iNOS (Figs. 1C and 2A), nitrotyrosine (Figs. 1E and 2A) and for anti-poly (ADP-ribose) polymerase (Figs. 1G and 2A) was found in the gingivomucosal tissues from ligature-operated rats. PDTC (10 mg/kg, i.p/daily) abolished the staining for TNF-α, iNOS, nitrotyrosine and anti-poly (ADP-ribose) polymerase (Figs. 1B. D, F, H and 2A respectively).

3.2. Effects of PDTC on plasma extravasation and neutrophils infiltration in periodontitis

Before the measurement of Evans blue extravasation, mean arterial pressure of vehicle-treated and PDTC-treated animals was recorded. In agreement with previous studies (Chatterjee et al., 2003), PDTC treatment did not affect mean arterial blood pressure (vehicle-treated: 128+6 mm Hg; $N=10$ and PDTC-treated: 125+7 mm Hg; $N=10$). Ligation significantly increased Evans blue extravasation in gingivomucosal tissue

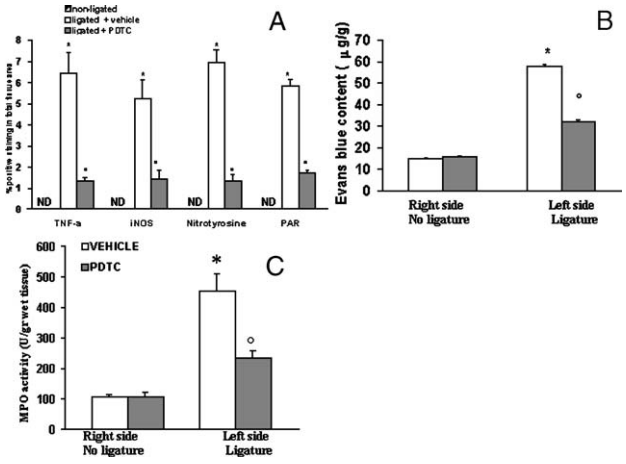

Fig. 2. Densitometry analysis of Immunocytochemistry photographs (A; $N=5$ photos from each samples collected from all rats in each experimental group) for TNF-α, iNOS, PAR and nitrotyrosine from gingivomucosal tissue was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Evans blue content (B) and Myeloperoxidase activity (C) in gingivomucosal tissue was significantly increased by ligature compared to the contralateral side. PDTC (10 mg/kg i.p., daily for eight days) significantly reduced Evans blue content and myeloperoxidase activity levels. Densitometry data are expressed as % of total tissue area. Data are means of mean±s.e.m. from $N=10$ rats for each group. $^{*}P<0.01$ vs. non-ligated. $^{\circ}P<0.01$ vs. ligated.

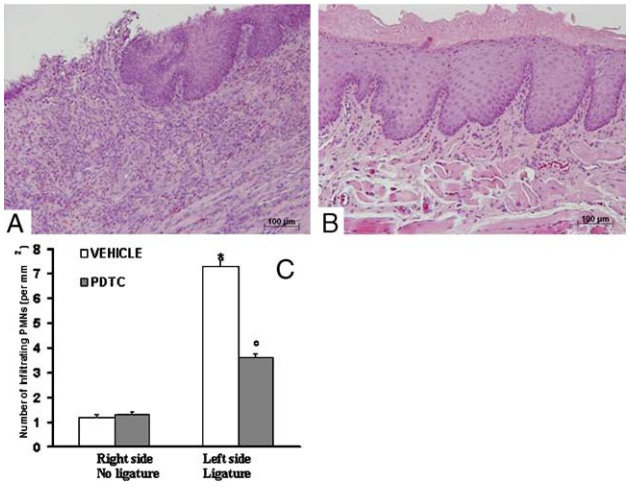

Fig. 3. Inflammatory cells infiltration and oedema were observed in gingivomucosal section from ligature-treated rats (A). Significantly less oedema and inflammatory cell infiltration was observed in gingivomucosal sections from ligature-treated rats which had been treated with PDTC (10 mg/kg i.p., daily for eight days) (B). The total number of infiltrating leukocytes (e.g., neutrophils and mononuclear cells) in gingivomucosal tissue was assessed quantitatively by counting the number of polymorphonuclear cell in 20 high-power fields (C). Figure is representative of at least 3 experiments performed on different experimental days. The tissue sections, orientated longitudinally from the teeth crown, were stained with trichrome stain. Data represent the mean±S.E.M. for 20 counts obtained from the gingivomucosal tissue of each treatment group. $^{*}P<0.01$ vs. non-ligated. $^{\circ}P<0.01$ vs. ligated.

compared to the contralateral side (Fig. 2B). PDTC treatment prevented this increase in Evans blue extravasation, but did not change the Evans blue content of the contralateral side (Fig. 2B). Myeloperoxidase activity was significantly elevated ($P<0.001$) at eight days after the ligature (Fig. 2C) and PDTC-treatment significantly reduced these levels (Fig. 2C). No significant changes of myeloperoxidase activity were observed in the gingivomucosal tissues from the contra lateral side (Fig. 2C).

3.3. Effect of PDTC on tissue damage and bone destruction

When compared to gingivomucosal tissue sections taken from the contra lateral side (data not shown), histological examination of gingivomucosal tissue sections of ligature-operated rats showed oedema, tissue injury as well as infiltration of the tissue with inflammatory cells (Fig. 3A). PDTC treatment reduced the degree of gingivomucosal tissues injury (Fig 3B). Quantification of infiltrating polymorphonuclear cell into gingivomucosal tissue showed that there were only a minimal number of polymorphonuclear cells in tissue from the contra lateral side (Fig. 3C). However, a large number of infiltrating polymorphonuclear cell were observed in the gingivomucosal tissue of ligated rats (Fig. 3C). PDTC administration significantly reduced the numbers of polymorphonuclear cell infiltrating into gingivomucosal tissue (Fig. 3C). A radiographic examination of the mandibles, at eight day after ligature placement, revealed bone matrix resorption in the lower left first molar region after ligation (Fig. 4A). There was no evidence of pathology in right first molar (data not shown). PDTC markedly reduced the degree of bone resorption in the lower left first

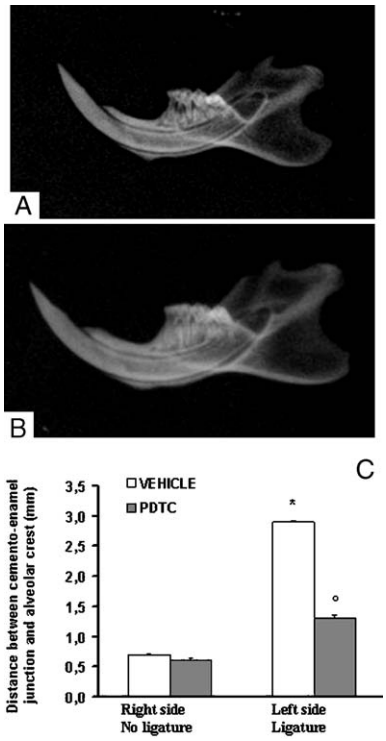

Fig. 4. The alveolar bone from ligated (8 days) rats demonstrated alveolar bone resorption (A). PDTC treatment suppressed alveolar pathology in the rat alveolar bone (B). A significant increase in the distance between cemento-enamel injunction and alveolar crest at mediolingulal root of the first molar was observed in ligature-treated rats. PDTC treatment significantly reduced the increase in the distance between cemento-enamel injunction and alveolar crest. Radiographic figure is representative of at least 3 experiments performed on different experimental days. Data represent the data from 20 counts obtained from the gingivomucosal tissue of each treatment group. *$P<0.01$ vs. non-ligated. °$P<0.01$ vs. ligated.

molar region after ligation (Fig. 4B). In addition, a significant alveolar bone loss, between the lower first left and the right first molars induced by the left side ligature, was observed in vehicle treated rats (Fig. 4C). PDTC treatment resulted in a significant inhibition of alveolar bone loss after ligation (Fig. 4C).

4. Discussion

In the present study, we demonstrate that PDTC reduces (i) the development of ligature-induced periodontitis, (ii) the infiltration of the gingivomucosal tissues with polymorphonuclear cells, (iii) the degree of nitrotyrosine formation in the gingivomucosal tissues and (iv) the degree of gingivomucosal tissues injury in rats' subjected to ligature-induced periodontitis. All of these findings support the view that PDTC attenuates the degree of experimental periodontitis in the rat.

Our results demonstrated that PDTC exerted a significant inhibitory effect on plasma extravasation 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 eighth day, than on the opposite side (Gyorfi et al., 1994). In addition, we also report in the present study that ligature-induced peridontitis in the rat results in a significant infiltration of inflammatory cells in the gingivomucosal tissues and we also demonstrated that treatment with PDTC reduces this inflammatory cells infiltration as assessed by myeloperoxidase and with the moderation of the tissue damage as evaluated by histological examination. Neutrophils are recruited into the tissue and can then contribute to tissue destruction by the production of reactive oxygen metabolites that further amplify the inflammatory response by their effects on macrophages and lymphocytes (Salvemini et al., 2001). A possible mechanism, by which PDTC attenuates polymorphonuclear cells infiltration, is by down-regulating adhesion molecules ICAM-1 and P-selectin as previously demonstrated (Cuzzocrea et al., 2002). These findings are in accordance with those of Berglundh and Lindhe (1993) who also found a significant increase in inflammatory cell infiltration in inflamed gingival as compared to a healthy one.

PDTC and other dithiocarbamates inhibit the activation of NF-κB and possess antioxidative properties (Ziegler-Heitbrock et al., 1993; Satriano and Schlondorff, 1994; Schwartz et al., 1996; Liu et al., 1997).Recently it has been suggested that the activation of NF-κB may also be under the control of oxidant/antioxidant balance (Cuzzocrea et al., 2004). Although, PDTC is an antioxidant, recent evidence suggests that this property may not be responsible for its ability to inhibit NF-κB in tubular epithelial cells (Woods et al., 1999). Paradoxically, the pro-oxidant and metal-chelating properties of PDTC could also be involved in its ability to inhibit NF-κB (Pinkus et al., 1996).

Activation of the transcription factor NF-κB is critical for the tumour necrosis factor-alpha (TNF-α)-induced inflammatory response and expression of inducible nitric oxide synthase (iNOS).

There is good evidence that TNFα helps to propagate the extension of periodontitis (Ikezawa et al., 2005). Nemeth and colleagues have previously reported that PDTC inhibits TNF-α production in an experimental model of endotoxic shock (Nemeth et al., 1998). Therefore, the inhibition of the production of TNF-α by PDTC described in the present study is most likely attributed to the inhibitory effect on the activation of NF-κB.

Moreover, several studies also support the conclusion that NO produced later on to activation of the iNOS play important roles in the pathogenesis of periodontitis (Di Paola et al., 2004). This study demonstrates that PDTC attenuates the expression of iNOS in periodontal tissue. Our finding of a reduced iNOS expression by PDTC in vivo are also in accordance with our recent reports that have clearly demonstrated the PDTC inhibits the expression of iNOS another model of inflammation (Cuzzocrea et al., 2002). Thus, the reduction of the expression of iNOS by PDTC may contribute to the attenuation by this agent of the formation of nitrotyrosine in the periodontal tissues from ligated-treated rats. ROS and peroxynitrite produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. ROS produce strand breaks in DNA that triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARP resulting in the activation of "the PARP Suicide Hypothesis". There is recent evidence that the activation of PARP may also play an important role in experimental periodontitis (Lohinai et al., 2003). We demonstrate here that

PDTC attenuates the increase in PARP activity in the periodontal tissue. In conclusion, our results indicate that PDTC has strong anti-inflammatory properties resulting in a reduced: 1) TNF-α production, 2) PMN infiltration, 3) the expression of iNOS, and ultimately the degree of peroxynitrite formation and tissue injury. The exact made of action of PDTC, however, still remains to be determined.

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