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H_2O_2 activates matrix metalloproteinases through the nuclear factor kappa B pathway and Ca^{2+} signals in human periodontal fibroblasts

C. Osorio[1], F. Cavalla[1,2], A. Paula-Lima[3], G. Díaz-Araya[4], R. Vernal[1,2], P. Ahumada[2], J. Gamonal[1,2], M. Hernández[2,5]

[1]Department of Conservative Dentistry, Faculty of Dentistry, Universidad de Chile, Santiago, Chile, [2]Laboratory of Periodontal Biology, Faculty of Dentistry, Universidad de Chile, Santiago, Chile, [3]Institute for Research in Dental Sciences, Universidad de Chile, Santiago, Chile, [4]Department of Chemical Pharmacology and Toxicology, Faculty of Chemistry and Pharmaceutical Sciences, Universidad de Chile, Santiago, Chile and [5]Department of Pathology, Faculty of Dentistry Universidad de Chile, Santiago, Chile

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Background: The mechanisms involved in reactive oxygen species and matrix metalloproteinase (MMP)-mediated periodontal tissue breakdown are unknown.

Objective: To determine the effect of H_2O_2 in MMP-2 and MMP-9 activity, and the involvement of nuclear factor kappa B (NFκB) and Ca^{2+}-mediated signals in human periodontal ligament fibroblasts.

Material and Methods: Primary cultures were characterized for their phenotype and exposed for 24 h to sublethal doses (2.5–10 μM) of H_2O_2 or control media. NFκB involvement was evaluated through immunofluorescence of p65 subunit, using the NFκB blocking peptide SN50 and catalase. Ca^{2+} signals were analyzed by loading the cells with Fluo4-AM and recording the fluorescence changes in a confocal microscope before and after the addition of H_2O_2. 1,2-bis (o-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl was used to chelate intracellular Ca^{2+}. The activity and levels of MMP-2 and MMP-9 were analyzed by gelatin zymogram and densitometric scanning, and enzyme-linked immunosorbent assay, respectively. Statistical analysis was performed with STATA V11.1 software using the ANOVA test.

Results: H_2O_2 at concentrations of 2.5–5 μM induced Ca^{2+} signaling and NFκB subunit p65 nuclear translocation, whereas catalase, SN50 and BAPTA-AM prevented p65 nuclear translocation. H_2O_2 at 2.5–5 μM significantly increased MMP-9 and MMP-2 activity, while SN50 resulted in lower MMP-2 and MMP-9 activity rates compared with controls.

Conclusion: Sublethal H_2O_2 induces Ca^{2+}-dependent NFκB signaling with an increase in MMP gelatinolytic activity in human periodontal ligament.

Marcela Hernández Ríos, DDs, MSc, PhD, Specialist in Oral Pathology, Avenida Sergio Livingstone 943, Comuna de Independencia, Santiago 8380492, Chile
Tel: +56 2 28781810
Fax: +56 2 27779724
e-mail: mhernandezrios@gmail.com

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Periodontal diseases involving both apical and marginal periodontium are characterized by a chronic immune inflammatory response to bacteria organized in the subgingival biofilm, which ultimately leads to the loss of hard and soft periodontal supporting tissues (1). An oxidative imbalance in favor of pro-oxidants is thought to play a key role in the loss of periodontal support and might be a common link between periodontitis and systemic diseases (2). ROS-mediated defense against bacterial invasion might result in the damage of host cell components, such as lipids, proteins and DNA, resulting in cell death. During the inflammatory process physiologic peroxide concentrations in the extracellular milieu are reported to be at micromolar levels (3,4). *In vitro* studies have proposed that non-toxic low concentrations of ROS might instead modulate the function of redox-sensitive proteins, such as the transcription factor NFκB, Ca^{2+} membrane channels and enzymes, but their molecular targets markedly differ among the various cell types (1,5).

Recent evidence of an oxidative activation of MMPs is also emerging (6,7). While MMP-2 and MMP-9 are recognized as key effectors of periodontal tissue breakdown and bone resorption (8–13), and might be involved in systemic complications (14,15), an association between ROS levels and these gelatinolytic MMPs has been suggested from clinical studies in periodontal diseases, but the nature of this association is not yet understood.

hPDL fibroblasts reside in direct relationship with mineralized tissues and are thought to play a key role in the homeostasis of periodontal tissues. Under bacterial and proinflammatory stimuli, such as bacterial lipopolysaccharide (LPS), tumor necrosis factor-α and interleukin-1β, they are able to synthesize considerable amounts of MMP-2 and MMP-9 (16). However, it is unknown whether hPDL fibroblasts respond to mild oxidative conditions and the potentially involved intracellular signals. The aim of this study was to evaluate the effect of H_2O_2 on MMP-2 and -9 levels and activity, and the potential involvement of NFκB and Ca^{2+} signals in hPDL fibroblasts.

Material and methods

Human periodontal ligament fibroblast primary cultures

hPDL were obtained from four patients (three females and one man) with a mean age of 24.5 years. All the patients attended the clinic of surgery at the Faculty of Dentistry, University of Chile. Systemically healthy patients over 18 years old were selected if they had indicated extraction of healthy fully erupted third molars with complete root formation. Pregnant women and patients who underwent antibiotic or non-steroidal anti-inflammatory treatment over the last 3 months were excluded. Subjects were recruited and clinical data were recorded for all participants with the understanding and written consent of each subject. The study was previously reviewed and approved by the Institutional Board of the Faculty of Dentistry in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki.

The extraction of hPDL was performed by the explant method (17). After reaching confluence, cells were digested (0.08% trypsin and 0.04% ethylenediaminetetraacetic acid) (Gibco, Invitrogen Co., Carlsbad, CA, USA), counted and plated. Primary cultures were used between passages 6 and 7 and three independent experiments were performed in triplicate. The supernatants were frozen at −80°C for later use.

Phenotypic characterization of human periodontal ligament

Primary cultures were characterized by morphological analysis in a phase contrast microscope (MicroImaging; Carl Zeiss Inc., Thornwood, NY, USA) and immunofluorescence. For the phenotypic immunofluorescence analysis, cells were fixed in 4% formaldehyde for 15 min at 4°C, permeabilized with Triton X-100 0.2% for 5 min (Gibco, Invitrogen Co.), blocked with bovine serum albumin (BSA) 3% (Gibco, Invitrogen Co.) at 4°C and incubated overnight with primary monoclonal antibodies anti-vimentin 1 : 100 (Novocastra Lab Ltd., Newcastle, UK), anti-periostin 1 : 100 (Merck KGaA, Calbiochem, Darmstadt, Germany) and anti-pan-cytokeratin 1 : 100 (Novocastra Lab Ltd.) diluted in 3% BSA, and the respective secondary antibodies. The samples were examined in a confocal microscope (MicroImaging; Carl Zeiss Inc.).

Exposure to H_2O_2

hPDL fibroblasts were seeded at 6×10^5 density until they reach confluence and then starved for 24 h to synchronize the cellular cycles. Cell viability was determined by trypan blue exclusion and 96 AQueous CellTiter commercial kit, according to the manufacturer's instructions (Promega, Madison, WI, USA). After testing different peroxide concentrations the cells were treated with sublethal doses of 10, 5 and 2.5 μM H_2O_2 for 24 h in Dulbecco minimal Eagle's medium (DMEM) solution with fetal bovine serum (FBS) 10% and conditioned medium was then replaced with DMEM without FBS for another 24 h.

Nuclear factor kappa B signaling

To evaluate the effect of H_2O_2 in the NFκB signaling pathway, the cellular localization of the NFκB subunit p65 was determined by immunofluorescence as described, using primary monoclonal anti-p65 antibody 1 : 50 in 3% BSA (Cell Signaling Technology, Inc., Beverly, MA, USA) and secondary antibody conjugated with Alexa 448 dye, and examined under a confocal microscope (MicroImaging; Carl Zeiss Inc.). Cells were treated with sublethal concentrations or no H_2O_2 in DMEM for 2 h. Additional controls included H_2O_2 5 μM/catalase 100 IU/mL (LLC; Sigma Aldrich Co., St. Louis, MO, USA), H_2O_2 5 μM/NFκB inhibitor of translocation peptide SN50 18 μM (Merck Biosciences,

Calbiochem), *Escherichia coli*'s LPS 10 ng/mL (LLC; Sigma Aldrich Co.), and LPS with SN50 18 μM.

Determination of intracellular Ca^{2+} signals

To evaluate whether H_2O_2 induced intracellular Ca^{2+} signals that might trigger NFκB nuclear translocation, cells were transferred to modified Tyrode solution (in mM: 129 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 glucose, 25 HEPES-Tris, pH 7.3), preloaded for 30 min at 37°C with 5 μM Fluo4-AM and washed three times with modified Tyrode solution to allow complete dye de-esterification. Fluorescence images of intracellular Ca^{2+} signals in hPDL were obtained every 15 s in an inverted confocal microscope (Axiovert 200, LSM 5 Pascal; Carl Zeiss, Jena, Germany; Plan Apochromatic 63 × Oil DIC objective, excitation 488 nm, argon laser beam). Frame scans were averaged using the equipment data acquisition program. Ca^{2+} signals are presented as F/F_0 values. All experiments were done at room temperature (20–22°C).

Matrix metalloproteinase determinations

To determine the effect of H_2O_2 in MMP activity and levels, the PDL fibroblasts were seeded at a density of 6×10^5 cell density in 100 mm petri dishes. Cells were washed and incubated in DMEM with 10% FBS plus H_2O_2 in 10, 5 and 2.5 μM for 24 h. DMEM with FBS 10% was used as control. To evaluate the influence of NFκB pathway in MMP-2 and -9 activity, a 5 μM H_2O_2/SN50 control was included. Supernatants from murine monocyte/macrophage RAW 264.7 (ATCC® TIB-71™) cells exposed to 10 ng/mL of LPS of *Escherichia coli* 0111:B4 (Fluka; Sigma-Aldrich Chemie, Buchs, Switzerland) were loaded as positive controls for gelatinolytic bands. After 24 h of exposure, conditioned medium was replaced by DMEM without 10% FBS for an additional 24 h as previously performed (16) and the supernatants were analyzed.

Gelatinolytic activity and the activity rate (ratio between active form vs. the sum of the pro-form and the active form) of MMP-2 and -9 were evaluated by gelatin zymography and quantified using the software "Gel logic pro" (Carestream Health, Rochester, NY, USA) and expressed as density units (du). A standard volume (8 μL) of culture supernatants was run under non-reducing denaturing conditions in 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis gels containing 1 mg/mL gelatin as substrate as previously reported (11). Total MMP-2 and MMP-9 levels were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Results were standardized per mg of protein, measured by Bradford's method.

Statistical analysis

Statistical analysis was performed using STATA V11.1 software (StataCorp, College Station, TX, USA). The distribution was determined using the Shapiro–Wilk's test. The results were analyzed using one-way ANOVA and Bonferroni *post-hoc* tests. $p < 0.05$ was considered statistically significant.

Results

Identification of primary cultures as human periodontal ligament fibroblasts

Cultured cells showed fibroblast-like morphology and no growth inhibition upon contact (data not shown). Cells were consistent with hPDL demonstrating immunopositivity for vimentin, α-actin, periostin and no staining for pancytokeratin (Fig. 1).

Sublethal concentrations of H_2O_2 in the viability of human periodontal ligament

As previously determined, working concentrations ≤ 10 μM did not show statistical differences ($p > 0.05$) in cell viability (Fig. 2A and 2B) or cell morphology (Fig. 2C), and were confirmed as sublethal for subsequent experiments.

H_2O_2 triggers the activation of nuclear factor kappa B signaling

The intracellular distribution of p65 subunit of NFκB in H_2O_2-stimulated hPDL was analyzed (Fig. 3). p65 subunit in the control group was predominantly located to the cytoplasm. Exposure to 2.5 μM H_2O_2 induced partial mobilization of the p65 subunit to the nucleus, whereas 5 μM H_2O_2 resulted in evident p65 nuclear translocation in a similar manner to the positive LPS control. In contrast, 10 μM H_2O_2 did not induce translocation of p65, but a perinuclear distribution. The addition of catalase, as well as the NFκB inhibitor peptide, SN50, prevented p65 nuclear translocation.

Nuclear translocation of nuclear factor kappa B depends on intracellular Ca^{2+} signals generated by H_2O_2

Representative fluorescence images from control hPDL cells loaded with fluorescent Ca^{2+} indicator Fluo4-AM, showed no significant changes in cytoplasmic fluorescence for 1500 s (Fig. 4A, Movie S1); otherwise, addition of 5 μM H_2O_2 to hPDL cells produced a rapid and sizable increase in cytoplasmic free [Ca^{2+}] (Fig. 4A, Movie S2). A representative average fluorescence trace (Fig. 4B) shows that this [Ca^{2+}] increase lasted several minutes before slightly decaying. On average, addition of H_2O_2 produced maximum values of $F/F_0 = 1.83 \pm 0.09$ (average of one to three cells per experiment, $n = 6$), while control cells exhibited no significant increases in the Fluo4 fluorescence. To investigate whether this intracellular Ca^{2+} rise induced by H_2O_2 was required for NFκB subunit p65 nuclear translocation, hPDL cells were preincubated for 30 min with 100 μM BAPTA-AM to chelate intracellular Ca^{2+}. As shown, NFκB subunit p65 nuclear translocation was evident at 5 μM H_2O_2. On the other hand, pre-treatment with BAPTA-AM nearly completely abrogated NFκB

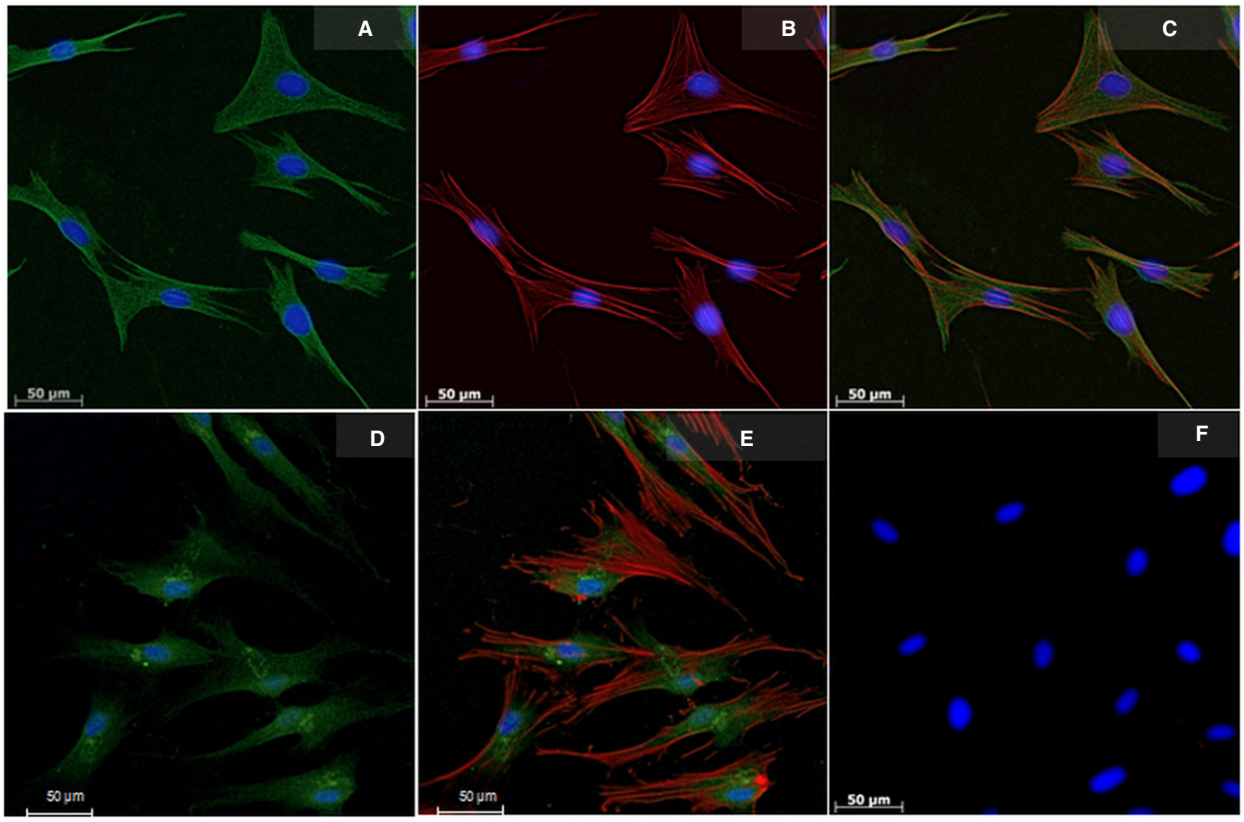

Fig. 1. Human periodontal ligament phenotypic characterization. The figure shows a representative image of three independent experiments. Immunolocalization of VIM, α-actin, VIM/α-ACT, PN, PN/α-ACT colocalization and PCK. α-ACT, α-actin; PCK, pancytokeratin; PN, periostin; VIM, vimentin.

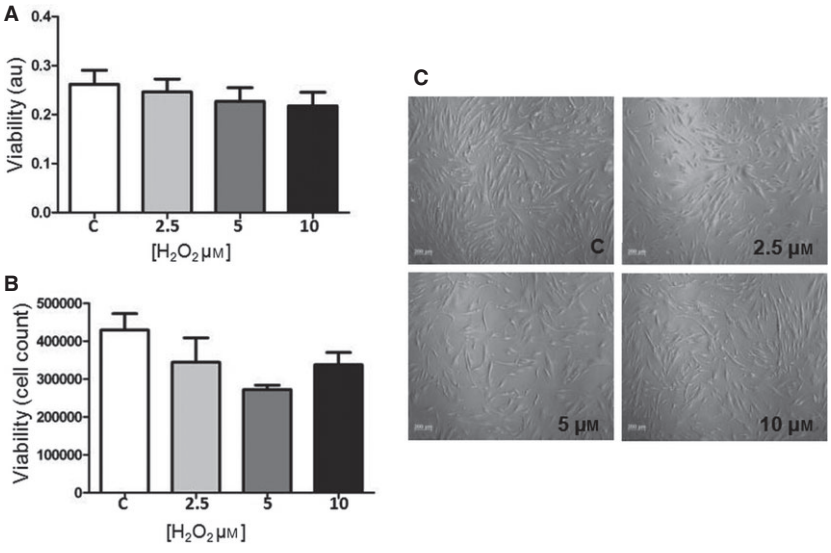

Fig. 2. Viability of human periodontal ligament exposed to H_2O_2. (A) MTS assay; (B) trypan blue exclusion; (C) human periodontal ligament fibroblast morphology at 5 ×. The figure shows a representative image of three independent experiments. Data are expressed as average ± SD. (C) H_2O_2 unexposed controls; 2.5, 5 and 10 represent H_2O_2-exposed cells expressed at μM concentrations. $p > 0.05$. au, arbitrary units.

subunit p65 nuclear translocation induced by 5 μM H_2O_2 (Fig. 4C).

H_2O_2 activates matrix metalloproteinase-2 and -9 with involvement of the nuclear factor kappa B pathway

Gelatinolytic bands were identified as MMP-9 pro-form (~92 kDa), active MMP-9 (~85 kDa), the pro-form of MMP-2 (~72 kDa) and its corresponding active form (~64 kDa) in H_2O_2-exposed groups and controls (Fig. 5A).

The gelatinolytic activity of proMMP-9 did not have any statistically significant variation in response to H_2O_2 exposure; however, it showed a marked tendency to increase at 2.5 and 5 μM peroxide stimulation (Fig. 5B). Active MMP-9 was significantly higher when exposed to 2.5 μM H_2O_2 in comparison to 10 μM and 5 μM/SN50, but no significant differences were

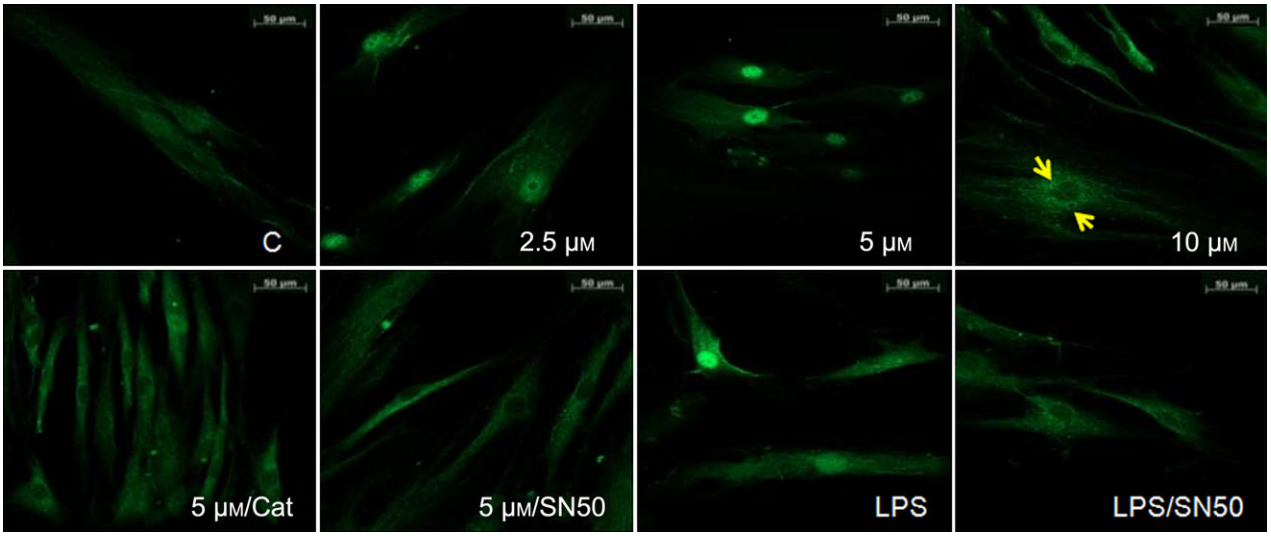

Fig. 3. p65 subunit immunolocalization in human periodontal ligament exposed to H_2O_2. Cells were unexposed (C) or treated with 2.5, 5 and 10 μM H_2O_2; 5 μM/CAT, 5 μM H_2O_2/SN50 18 μM, LPS 2 μg/mL and LPS/SN50. The figure shows a representative image of three independent experiments, magnification 40 ×. Arrows show perinuclear distribution of p65 at 10 μM peroxide concentration. C, control; 5 μM/CAT, 5 μM H_2O_2/catalase; LPS, lipopolysaccharide; LPS/SN50, LPS 2 μg/mL plus SN50.

observed with unexposed controls ($p = 0.0027$; Fig. 5C). The activation rate observed for MMP-9 was borderline reduced in the 5 μM/SN50 group compared to the controls ($p = 0.05$, Fig. 4D).

No differences were observed in proMMP-2 gelatinolytic activity among H_2O_2-exposed and non-exposed groups (Fig. 5E), whereas a significant increase in active MMP-2 was evident for 5 μM H_2O_2 in comparison with unexposed controls, 10 μM H_2O_2 and 5 μM/SN50 (Fig. 5F). The activation rate of MMP-2 demonstrated a significant increase when exposed to 5 μM H_2O_2 compared to the control with no peroxide ($p = 0.0002$; Fig. 5G). No differences were observed for total MMP-2 and -9 levels ($p = 0.49$ and 0.08, respectively; Fig. 5H and 5I).

Discussion

In contrast to the widespread known role of ROS as triggers for oxidative cell damage, low physiologic concentrations of ROS can regulate a variety of cell functions that may be relevant to periodontal homeostasis and inflammation (18,19). Non-toxic low H_2O_2 concentrations can modify the intracellular redox state, inducing modifications of cell signaling targets (5,6,20,21). This study evaluated the effect of non-toxic low H_2O_2 in hPDL, focusing on the activity of two relevant effector enzymes in periodontitis, MMP-2 and -9, and the involvement of NFκB and Ca^{2+} signaling in primary cultures of hPDL undergoing low oxidative stress through H_2O_2.

Most studies assess the effects of H_2O_2 in high concentrations, ranging in the order of mM (6,21). Nevertheless, previous reports evidence that physiologic concentrations of H_2O_2 in the interstitium of inflamed periodontal tissues and activated phagocytes range in the order of μM (11,22). In line with this, we evaluated low H_2O_2 concentrations in primary cultures consistent with hPDL fibroblasts. Concentrations up to 10 μM maintained cell viability and morphology in the present study.

The exposure of hPDL to low sublethal doses up to 5 μM H_2O_2 resulted in a dose-dependent activation of the NFκB pathway, as demonstrated by nuclear translocation of the p65 subunit that was prevented by catalase, further supporting the role of H_2O_2 as a second messenger in hPDL. Conversely, H_2O_2 at 10 μM did not induce the p65 nuclear translocation, but its perinuclear accumulation. Previous works indicate that exogenous H_2O_2 generates intracellular Ca^{2+} signals and activation of transcription factors, including NFκB in rat nervous cells and human dermal fibroblasts; however, a high variability of responses has been reported according to cell type (23–25). Accordingly, H_2O_2 induced Ca^{2+}-dependent signaling at concentrations similar to this study in osteoclast-like cells (5), whereas concentrations from 20 μM induced senescence of gingival fibroblasts from mice (26).

hPDL fibroblasts reside in close relationship with mineralized tissues, regulating their homeostasis. Extracellular Ca^{2+} has been proposed to play a role as first messenger in hPDL fibroblasts (27), and to regulate mineralized tissue turnover and inflammation with the involvement of NFκB signaling in murine cementoblasts. On the other hand, hPDL cells express T-type Ca^{2+} channels and intracellular Ca^{2+} signals in response to hydraulic pressure and specific growth factors, hormones and cytokines (28,29).

In line with these antecedents, we investigated if H_2O_2 induces Ca^{2+} signals in hPDL primary cultures and whether Ca^{2+} signals were involved in activation of the NFκB pathway in these cells. Our results indicate that 5 μM H_2O_2 induced a rapid and sizable increase in cytoplasmic free [Ca^{2+}] in

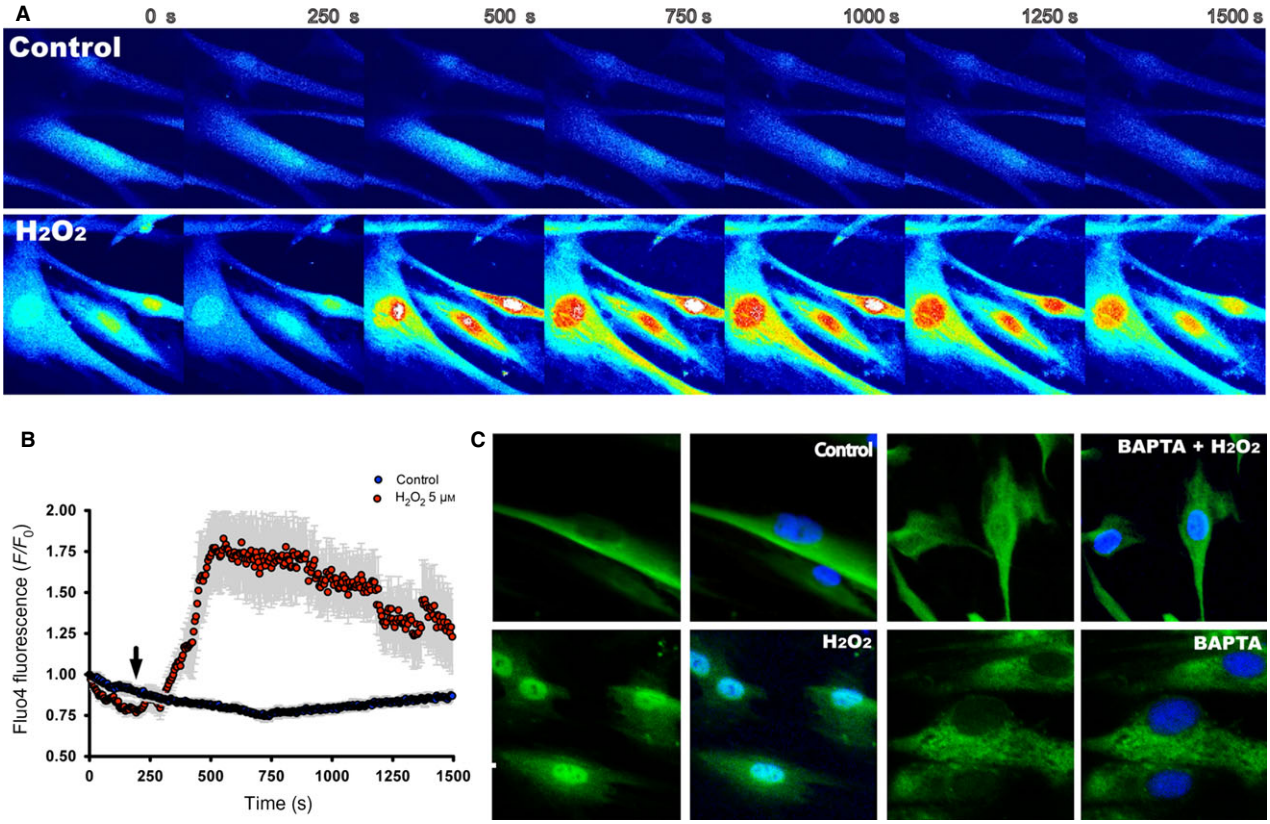

Fig. 4. Determination of Ca^{2+} signals in FLP cells exposed to H_2O_2 and its role on p65 subunit translocation. (A) Representative images of confocal sections obtained from control fibroblasts loaded with Fluo4-AM and recorded before (top line) and after (bottom line) addition of 5 µM H_2O_2. Time-dependent changes in Fluo4 fluorescence intensity were shown in pseudo-color images ranging from lower intracellular [Ca^{2+}] (blue) to higher intracellular [Ca^{2+}] (red). (B) Representative time course of Fluo4 fluorescence changes recorded before and after addition of 5 µM H_2O_2 to fibroblasts. Values represent mean ± SE (n = 3 independent cultures, 4–10 cells analyzed per culture). (C) p65 subunit immunolocalization in human periodontal ligament exposed to 10 µM BAPTA-AM previously to the addition of 5 µM H_2O_2. p65 immunostaining is observed as green fluorescence, while nuclear staining are observed in blue (Hoechst). Representative images of p65 staining are showed on the left side, while merge images with nuclear staining are showed on the right side. Magnification 60 ×.

hPDL, which did not reach basal levels until 1300 s after the addition of H_2O_2. Interestingly, incubation of the cells with BAPTA-AM, which chelates intracellular Ca^{2+}, partially abrogated p65 subunit translocation induced by H_2O_2, indicating that Ca^{2+} is involved in ROS-activated NFκB signaling in hPDL. Nevertheless, additional mechanisms controlling NFκB signaling in response to H_2O_2 in hPDL cannot be discarded, such as direct H_2O_2 activation.

The role of low concentrations of ROS in cell signal transduction is regulated through the presence of redox-sensitive cysteines, activating transcription factors, such as NFκB, and Ca^{2+} membrane channels, and inhibiting Ca^{2+}-extruding proteins, among others (18,20). The connection between H_2O_2, Ca^{2+} signals and NFκB has been previously described in cell types other than fibroblasts. Neuronal activity induces H_2O_2 generation that stimulates intracellular Ca^{2+} signals (30), which enhance nuclear translocation of the NFκB p65 protein and NFκB-dependent transcription (31). Herein, a cross-talk between H_2O_2, intracellular Ca^{2+} and NFκB pathway is reported in hPDL fibroblasts; H_2O_2-induced Ca^{2+} might potentiate the p65 NFκB nuclear translocation, either through direct means or through the enhancement of ROS synthesis. On the other hand, H_2O_2 has also been reported to induce cytotoxicity, resulting in perinuclear accumulation of p65/p55 (32). Thus, the effects of H_2O_2 on the NFκB pathway might be pleiotropic and even antagonistic, depending on the dose, interactions and/or cell type. Based on this, p65 perinuclear accumulation in response to 10 µM H_2O_2, might be interpreted as an early sign of cell toxicity.

Although there are no previous studies in the current cell system, and in line with our results, low doses of H_2O_2 have been reported to stimulate phosphorylation of the NFκB/inhibitor (I)κB complex. This enables the p65 subunit nuclear translocation facilitating its binding to the promoters of inflammatory cytokine genes (1) and oxidizing enzymes, such as MMPs (6,20). In turn, cytokines can further stimulate ROS production in phagocytic cells, contributing to their hyper-reactive phenotype during periodontitis (33). In the present study,

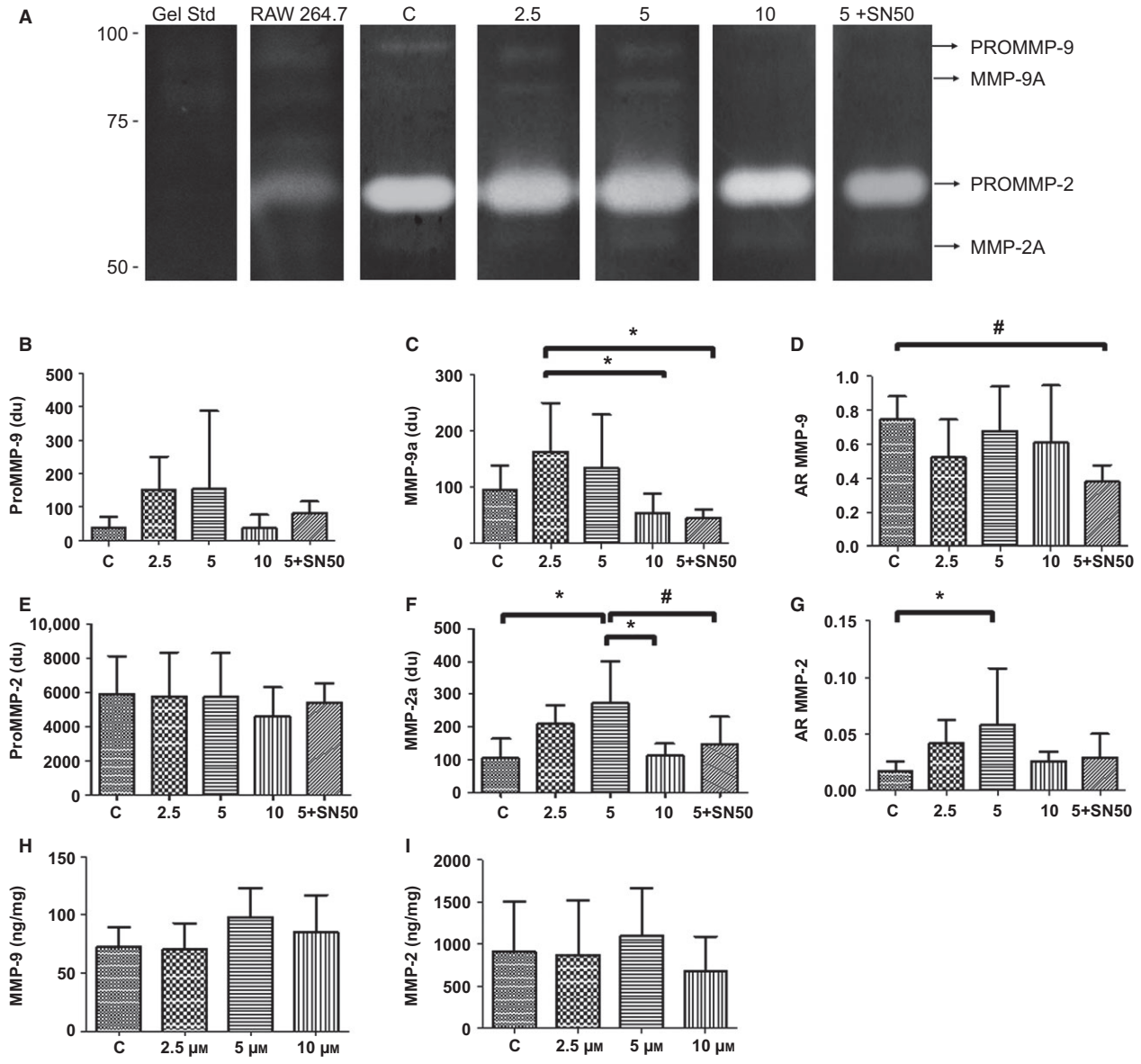

Fig. 5. MMP-2 and -9 gelatinolytic activity in human periodontal ligament exposed to H_2O_2. (A) identification of representative gelatinolytic MMP bands; (B) proMMP-9; (C) active MMP-9 (ANOVA, $p = 0.0027$); (D) AR of MMP-9 (ANOVA, $p = 0.047$); (E) proMMP-2; (F) active MMP-2 (ANOVA, $p = 0.0002$); (G) AR MMP-2 (ANOVA, $p = 0.0027$); (H) total MMP-9; (I) total MMP-2. $*p < 0.05$, $\#p = 0.05$. AR, activity ratio; C, H_2O_2 non-exposed controls; 2.5, 5 and 10 represent H_2O_2 exposure at µM concentrations; Du, densitometric units; GEL STD, gelatinase standard; MMP, matrix metalloproteinase; RAW 264.7, positive control from murine monocyte/macrophage RAW 264.7 cells exposed to 10 ng/mL of lipopolyssacharide of *Escherichia coli*.

when evaluating MMPs and involvement of the NFκB pathway, the exposure of hPDL to sublethal doses of H_2O_2 for 24 h did not influence proenzyme or total MMP-2, whereas proMMP-9 levels showed a marked tendency to increase at 2.5 and 5 µM peroxide stimulation, but they did not reach statistical significance. In contrast, active MMP-2 significantly increased with 5 µM H_2O_2; additionally, increasing of H_2O_2 up to 10 µM and inhibition of NFκB with SN50 decreased the MMP activity/activation rate to levels comparable to the controls. Altogether these results suggest that low doses of H_2O_2 are capable of activating NFκB, increasing gelatinolytic activity in hPDL, whereas higher concentrations might inhibit NFκB signaling and its downstream effects.

MMP activation by ROS is also supported by previous reports in other cell culture systems. Oxidative stress increases extracellular matrix turnover mediated by MMP-2, -9 and -13 in diploid fibroblasts, cardiac fibroblasts and tumor cells (6,20,34). Peroxide stimulus can increase the activity of MMP-2 and -9, without increasing MMP synthesis in fibroblastic cell lines (TIG-7).

In the same line, it was shown that *Aggregatibacter actinomycetemcomitans*-induced activation of MMP-2 could be blocked by an NFκB inhibitor, further supporting involvement of the NFκB signaling pathway (35).

The mechanisms involved in extracellular MMP activation are complex and may vary depending on the tissue type and disease. Previous studies revealed that proMMP-9 could be activated by MMP-13 in gingival tissue (36) along with proMMP-2 *in vitro* (37). ProMMP-2 might be activated in turn by MMP-9 and MT1-MMP forming thus an amplification loop involving the co-ordinated activation of MMP-13, MMP-2 and MMP-9 following MT1-MMP upregulation (37–39). The mechanisms supporting intracellular signaling-mediated MMP activation are even less clear. Activation of NFκB signaling in dermal fibroblasts induced MT1-MMP overexpression, which subsequently activated proMMP-2. This observation was further confirmed by the identification of a consensus p65 NFκB binding site in the human MT1-MMP gene promoter (40). Additionally, intracellular Ca^{2+} was shown to regulate MT1-MMP processing affecting down-stream MMP-2 activity (18). Altogether, the evidence points out to the induction of the MMP-2-mediated activation cascade via Ca^{2+} and NFκB-induced expression of MT1-MMP, although this remains to be proved in PDL fibroblasts.

Recent clinical studies reproduce the association between active MMPs and ROS. The active form of MMP-2 was shown to correlate positively with total oxidant status in apical lesions of endodontic origin (11), whereas total and active forms of MMP-9 and MMP-8 isoforms have been reported to correlate positively with myeloperoxidase levels in progressive chronic periodontitis (12), pointing towards an oxidative-dependent MMP activation via MPO-derived HOCl (41). Based on the current results, H_2O_2 is able to induce activation of the key MMPs in periodontal tissues, either by means of an indirect mechanism, through Ca^{2+} and NFκB-mediated intracellular signaling, or direct enzyme oxidation (42). Overall, this study provides the first evidence that non-toxic low doses of H_2O_2 induce MMP activity in hPDL, at least in part through a cross-talk between activation of the NFκB and Ca^{2+} signals, suggesting a mechanistic basis to explain the regulatory role of ROS in periodontal tissue homeostasis and breakdown.

Conclusion

Five micromolar H_2O_2 induces Ca^{2+} signaling and NFκB nuclear translocation along with an increase in MMP gelatinolytic activity in hPDL. Intracellular Ca^{2+} chelation partially abrogated p65 nuclear translocation, supporting that Ca^{2+} signals modulate the NFκB pathway. The MMP activity was reduced by NFκB inhibition, suggesting the participation of this signaling pathway. This finding contributes to understanding the roles of ROS and their related intracellular signals during periodontal inflammation, as well as to identify potential targets for future diagnostics and treatment.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Movie S1 and S2. RECORD of Ca^{2+} signals in FLP cells exposed to CONTROL VEHICLE (S1) AND 5 μM H_2O_2 (S2) by confocal microscopy. time-dependent changes in FLUO4-am fluorescence intensity were shown in pseudo-colour images ranging from lower intracellular [Ca^{2+}] (blue) to higher intracellular [Ca^{2+}] (red).

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