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Defense mechanism of heme oxygenase-1 against cytotoxic and receptor activator of nuclear factor-κB ligand inducing effects of hydrogen peroxide in human periodontal ligament cells

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Background and Objective: Although induction of heme oxygenase-1 by H_2O_2 has been reported, the protective role of heme oxygenase-1 against the cytotoxic and osteoclastogenic effects of H_2O_2 have not been elucidated in human periodontal ligament cells. The aim of this work was to investigate the defense mechanism of heme oxygenase-1 on H_2O_2-induced cytotoxicity and to analyze the expression of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin as markers for osteoclast differentiation in periodontal ligament cells.

Material and Methods: Using human periodontal ligament cells, cytotoxicity was measured by the 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay, and expression of heme oxygenase-1, RANKL, and osteoprotegerin mRNA was determined by reverse transcription–polymerase chain reaction.

Results: H_2O_2 produced a cytotoxic effect by reducing the cell viability and enhancing the expression of heme oxygenase-1 and RANKL mRNAs in a concentration- and time-dependent manner. Additional experiments revealed that heme oxygenase-1 inducer (hemin), a membrane-permeable cGMP analog (8-bromo-cGMP), carbon monoxide, extracellular signal-regulated kinase, p38 mitogen-activated protein kinase inhibitor, protein kinase inhibitor (KT5823), and nuclear factor-κB inhibitor (pyrrolidine dithiocarbamate) also blocked the effects of H_2O_2 on cell viability and RANKL mRNA expression in periodontal ligament cells.

Conclusion: These data suggest that heme oxygenase-1 induction plays a protective role in periodontal ligament cells against the cytotoxic and RANKL-inducing effects of H_2O_2, through multiple signaling pathways.

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Reactive oxygen species, including oxygen free radicals (e.g. superoxide anions, O_2^-, and hydroxyl radicals, OH·) and H_2O_2, have harmful effects on cells and tissues during inflammation and wound healing, particularly on cell membranes, DNA, proteins, and lipids (1,2). Moreover, osteoclasts have been shown to produce reactive oxygen species (3–5) and also to be activated by them (6,7). For these reasons, reactive oxygen species, such as superoxide and H_2O_2, have been proposed as key players in bone resorption, although the mechanisms by which reactive oxygen species mediate osteoclast differentiation and bone resorption are not completely understood.

The periodontal ligament is a highly vascularized and cellularized connective tissue that attaches the root of the tooth to the surrounding alveolar bone (8). The cells of the periodontal ligament exhibit some characteristics of osteoblasts, in that they support new bone formation *in vivo*, and periodontal ligament cultured with 1α,25-dihydroxyvitamin D_3 produces the receptor activator of nuclear factor-κB ligand (RANKL) (9). Because of these findings, periodontal ligament is now believed to be involved in the regulation of alveolar bone metabolism.

Receptor activator of nuclear factor-κB (RANK) and RANKL also play an important role in osteoclastogenesis and bone resorption (10). Osteoprotegerin, a decoy receptor for RANKL, inhibits osteoclastogenesis and suppresses bone resorption (11). Periodontal ligament cells secrete osteoprotegerin and inhibit osteoclastogenesis (12,13). RANKL has also been found in periodontal ligament cells in periodontal disease and during orthodontic tooth movement (14,15). These reports indicate that periodontal ligament cells synthesize both RANKL and osteoprotegerin, and that inactivation of osteoprotegerin may play a key role in periodontal ligament cell differentiation.

Heme oxygenase is the rate-limiting enzyme in heme catabolism. Its reaction products are equimolar quantities of carbon monoxide, free iron, and biliverdin; the latter two species are converted to ferritin and bilirubin, respectively (16). One of three mammalian heme oxygenase isoforms, heme oxygenase-1 (also called heat shock protein 32), is a stress-responsive protein induced by various agents and is involved in a variety of regulatory and protective mechanisms in cells (17–19).

We previously reported that heme oxygenase-1 is induced by pro-inflammatory cytokines (20) and nitric oxide (21), and may play a role in cytoprotection. Several studies have demonstrated that H_2O_2 induces heme oxygenase-1 gene expression in macrophages, mesangial cells, neuronal cells, muscle cells, endothelial cells, and human embryonic kidney cells (22–27); however, a protective role for heme oxygenase-1 against the cytotoxic and osteoclastogenic effects of H_2O_2 has not been reported in periodontal ligament cells.

The purpose of this study was to examine whether H_2O_2 regulates the osteoprotegerin and RANKL signaling pathways and cellular function through a heme oxygenase-1-dependent mechanism in periodontal ligament cells. In the present study, we demonstrate that exogenous H_2O_2 is cytotoxic and simultaneously induces heme oxygenase-1 and RANKL mRNA expression via the cGMP, mitogen-activated protein (MAP) kinase, protein kinase G, and nuclear factor-κB pathways in human periodontal ligament cells. We also show that the induced heme oxygenase-1 subsequently plays a protective role against H_2O_2 cytotoxicity and RANKL mRNA induction in these cells.

Material and methods

Reagents

Dulbecco's modified Eagle's medium, fetal bovine serum, and other tissue culture reagents were purchased from Gibco BRL (Grand Island, NY, USA). 1H-[1,2,4]oxadiazole[4,3-α]quinoxalin-1-one (ODQ) was acquired from Alexis (San Diego, CA, USA), and zinc protoporphyrin IX and hemin were purchased from Porphyrin Products (Logan, UT, USA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless indicated otherwise.

Primary culture of periodontal ligament

Periodontal ligaments were isolated, using an explant culture technique from patients undergoing orthodontic treatment, by previously described methods (28). Informed written consent from donors were obtained for use of the tissues. Patients signed the corresponding informed consent approved by the Institutional Review Board at Wonkwang University for use of the tissues. Briefly, these tissues were cut into 1-mm^2 explants and placed in a 100-mm culture dish (Nunc, Naperville, IL, USA) containing 10,000 U/mL of penicillin G sodium, 10,000 μg/mL of streptomycin sulphate, 25 μg/mL of amphotericin B, and 10% heat-inactivated fetal bovine serum (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 2 or 3 d, cells started to outgrow from the explants. When the primary cell culture reached confluence, cells were detached with 0.025% trypsin and 0.05% EDTA, diluted with culture medium, and then subcultured in a ratio of 1 : 4. Cell cultures between the 4th and 7th passages were used in this study.

Cell viability analysis

Viable cells were detected using 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) dye, which forms blue formazan crystals that are reduced by the mitochondrial dehydrogenase present in living cells. Briefly, 2×10^4 cells were seeded in a 96-well plate and cultured overnight for cell attachment. The cells were treated by adding serial dilutions of H_2O_2. Cultures of Dulbecco's modified Eagle's medium and 10% fetal bovine serum were used as a negative control. After treatment, 50 μL of MTT solution (2 mg/mL in phosphate-buffered saline) was added to each well, and the cells were incubated for 4 h. The plates were then centrifuged at 200 *g* for 10 min, the supernatant was discarded, and 50 μL of dimethylsulfoxide were added to each well. The plates were

shaken until the crystals had dissolved, and the reduced MTT was measured spectrophotometrically at 570 nm in a dual-beam microtiter plate reader.

RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR)

Cells were grown in 60-mm culture dishes and incubated for 4–24 h in a fresh medium containing stimuli, as indicated. After discarding the growth medium, total RNA was isolated from cells using easy-Blue (iNtRON Biotechnology, Daejon, Korea), following the manufacturer's instructions. RT of the RNA was performed using AccuPower RT PreMix (Bioneer, Daejon, Korea). One microgram of RNA and 20 pmol primers were pre-incubated at 70°C for 5 min and transferred to a mixture tube. The reaction volume was 20 μL. cDNA synthesis was performed at 42°C for 60 min, followed by RT inactivation at 94°C for 5 min. Thereafter, the RT-generated DNA (2–5 μL) was amplified using AccuPower PCR PreMix (Bioneer). The primers used for cDNA amplification were the same as in previous reports (13–15,29), as follows: osteoprotegerin (13), 575 bp, (forward) 5′-TGCAGTACGTCAAGCAGGAG-3′, (reverse) 5′-TGACCTCTGTGAAAACAGC-3′; RANKL (13), 605 bp, (forward) 5′-CCAGCATCAAAAYCCCAAGT-3′, (reverse) 5′-CCCCTTCAGATGATCCTTC-3′; heme oxygenase-1 (29), 399 bp, (forward) 5′-AAGATTGCCCAGAAAGCCCTGGAC-3′, (reverse) 5′-AACTGTGCCACCAGAAAGCTGAG-3′; and glyceraldehyde-3-phosphate dehydrogenase (13), 306 bp, (forward) 5′-CGGAGTCAACGGATTTGGTCGTAT-3′; (reverse) 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. PCR conditions were as follows. Osteoprotegerin and RANKL: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min in a DAN thermal cycler (model 480; Perkin Elmer, Wellesley, MA, USA). Heme oxygenase-1: 30 cycles at 94°C for 60 s, 60°C for 30 s, and 72°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase: 25 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s. PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide. The relative intensity of the gel bands was measured using QUANTITY-ONE software (Bio-Rad Co., Hercules, CA, USA), and results were normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase, a housekeeping enzyme. Experiments were performed in triplicate and the most representative experiment was selected for demonstration.

Western blot analysis

Protein samples (50 μg) were mixed with an equal volume of ×2 sodium dodecyl sulfate sample buffer, boiled for 5 min, and then separated through 8–15% sodium dodecyl sulfate–polyacrylamide gels. After electrophoresis, proteins were transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% dry milk (for 1 h), rinsed, and incubated with antibody to heme oxygenase-1 (diluted at 1 : 1000) in Tris-buffered saline overnight at 4°C. Primary antibody was then removed by washing the membranes four times in Tris-buffered saline, and labeled by incubating with 0.1 mg/mL peroxidase-labeled secondary antibodies (against mouse and rabbit) for 1 h. Following three washes in Tris-buffered saline, bands were visualized by enhanced chemiluminescence and exposed to X-ray film.

Statistical analysis

Differences among groups were analyzed using one-way analysis of variance combined with the Bonferroni test. All values were expressed as means ± standard deviations, and differences were considered significant at $p < 0.05$.

Results

Effects of H_2O_2 on cell viability and expression of osteoprotegerin, RANKL, and heme oxygenase-1 in periodontal ligament cells

Initially, we estimated the cytotoxic effect of H_2O_2 on human periodontal ligament cells using the MTT assay. When periodontal ligament cells were exposed to different concentrations of H_2O_2 for various lengths of time, cell viability was reduced in a concentration- and time-dependent manner, as compared with control cells (Fig. 1A,D). Exposure of cells to 1.0 or 2.0 mM H_2O_2 for 24 h showed a decrease in cell viability (Fig. 1A).

Levels of osteoprotegerin, RANKL, and heme oxygenase-1 mRNAs were assayed by RT-PCR amplification and subsequent electrophoresis on 1.5% agarose gels, which are shown in Fig. 1B,E. When periodontal ligament cells were treated with 1.0 mM H_2O_2 for 24 h, osteoprotegerin mRNA expression remained unchanged. Furthermore, 24 h of exposure to H_2O_2 at concentrations ranging from 0.1 to 2.0 mM also had no effect on osteoprotegerin mRNA expression. In contrast, RANKL mRNA expression was up-regulated by H_2O_2 in a time- and concentration-dependent manner up to 1.0 mM H_2O_2, but was nearly arrested at 2.0 mM H_2O_2 (Fig. 1B).

We examined human periodontal ligament cells for the expression of heme oxygenase-1 mRNA and protein induced by exogenous H_2O_2. When periodontal ligament cells were treated with H_2O_2, heme oxygenase-1 mRNA and protein expression increased in a time- and concentration-dependent manner (Fig. 1B,C,E,F); maximum levels of both heme oxygenase-1 mRNA and heme oxygenase-1 protein occurred at 1.0 mM H_2O_2, and they decreased at 2.0 mM H_2O_2. When the periodontal ligament cells were treated with 1 mM H_2O_2 until 48 h, the level of heme oxygenase-1 mRNA and protein reached their maximum levels after 24 h of exposure (Fig. 1E). The increase in heme oxygenase-1 protein appeared to correspond to levels of heme oxygenase-1 mRNA accumulation (Fig. 1C,F).

Effects of hemin and zinc protoporphyrin IX pretreatment on the cytotoxic and RANKL mRNA-inducing effects of H_2O_2 in periodontal ligament cells

To assess further the role of heme oxygenase-1 induction in counteracting

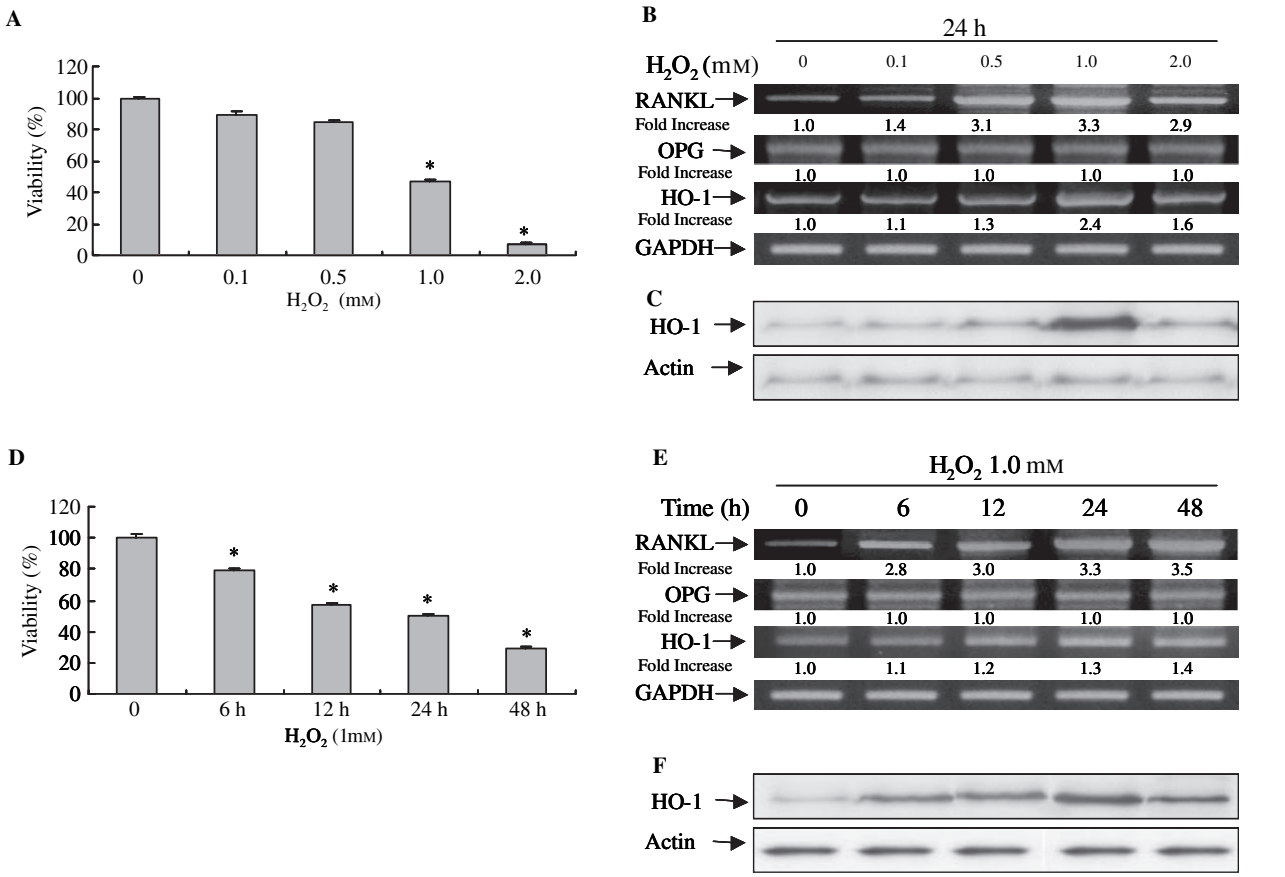

Fig. 1. Effect of H_2O_2 on cytotoxicity (A,D), expression of osteoprotegerin, receptor activator of nuclear factor-κB ligand (RANKL), heme oxygenase-1 mRNA (B,E) and protein expression (C,F) in periodontal ligament cells. Human periodontal ligament cells were incubated for 24 h with various concentrations of H_2O_2 (A,B,C) and 1 mM H_2O_2 for various durations (D,E,F). Cell viability test, mRNA and protein expression were determined by MTT, semiquantitative reverse transcription–polymerase chain reaction and Western blotting, respectively. Numbers below the gels represent the intensity of RANKL, osteoprotegerin and heme oxygenase-1 mRNA relative to glyceraldehyde-3-phosphate dehydrogenase mRNA. *Statistically significant difference compared with the control group, $p < 0.05$. Reverse transcription–polymerase chain reaction and Western blotting results are representative of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; OPG, osteoprotegerin.

the cytotoxic and RANKL mRNA-inducing effects of H_2O_2 on periodontal ligament cells, we investigated the effect of pretreatment of the cells with hemin, a heme oxygenase-1 inducer. Hemin pretreatment increased induction of heme oxygenase-1 expression by H_2O_2 and significantly reduced the cytotoxic and RANKL-inducing effects of H_2O_2 (Fig. 2A,B). The involvement of heme oxygenase-1 in cytoprotection against H_2O_2 was also confirmed using zinc protoporphyrin IX, a well-known heme oxygenase-1 inhibitor. Zinc protoporphyrin IX pretreatment blocked induction of heme oxygenase-1 expression by H_2O_2 and enhanced the cytotoxic and RANKL-inducing effects of H_2O_2 (Fig. 2A,B).

Effects of 8-bromo-cGMP and ODQ pretreatment on the cytotoxic and RANKL mRNA-inducing effects of H_2O_2 in periodontal ligament cells

To determine whether guanylate cyclase or cGMP are mediators of the cytotoxic and RANKL mRNA-inducing effects of H_2O_2, we exposed periodontal ligament cells to the cGMP analog, 8-bromo-cGMP. Pretreatment with 50 μM 8-bromo-cGMP for 12 h attenuated H_2O_2 cytotoxicity (Fig. 3A), decreased H_2O_2-induced expression of RANKL mRNA, and increased H_2O_2-induced expression of heme oxygenase-1 mRNA (Fig. 3B). In contrast, pretreatment of periodontal ligament cells with a selective inhibitor of guanylate cyclase, ODQ, decreased induction of heme oxygenase-1 expression by H_2O_2 and accentuated both the cytotoxic and RANKL-inducing effects of H_2O_2 (Fig. 3A,B).

Effects of CO and iron pretreatment on the cytotoxic and RANKL mRNA-inducing effects of H_2O_2 in periodontal ligament cells

To determine which of the heme cleavage products catalyzed by heme

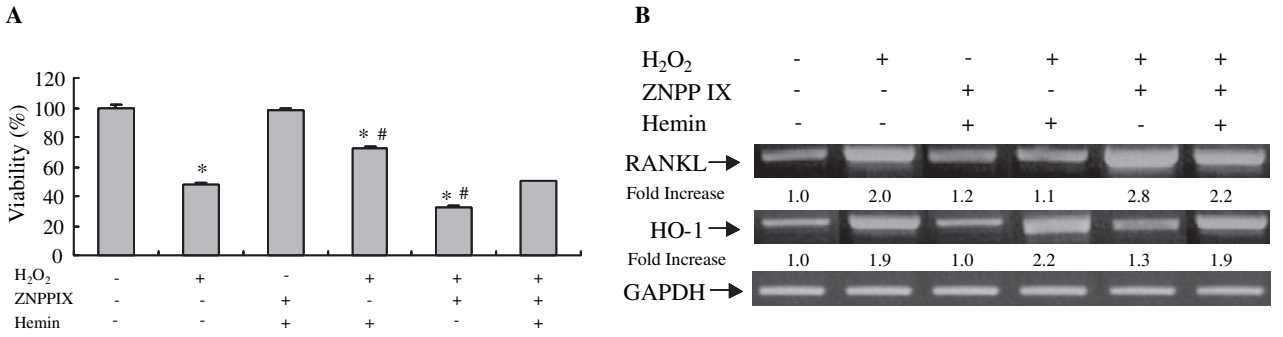

Fig. 2. Effects of hemin and the heme oxygenase-1 inhibitor, zinc protoporphyrin IX, on H_2O_2-mediated cytotoxicity (A), receptor activator of nuclear factor-κB ligand (RANKL), and heme oxygenase-1 mRNA expression (B). Cells were pretreated with hemin (10 μM) or zinc protoporphyrin IX (20 μM) for 12 h, and treated for 24 h with H_2O_2 (1.0 mM) in periodontal ligament cells. *Statistically significant difference compared with the control group, $p < 0.05$. Statistically significant difference compared with the H_2O_2 group, $p < 0.05$. The same procedure as described in the legend to Fig. 1 was performed. Results are representative of three independent experiments. HO-1, heme oxygenase-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ZNPPIX, zinc protoporphyrin IX.

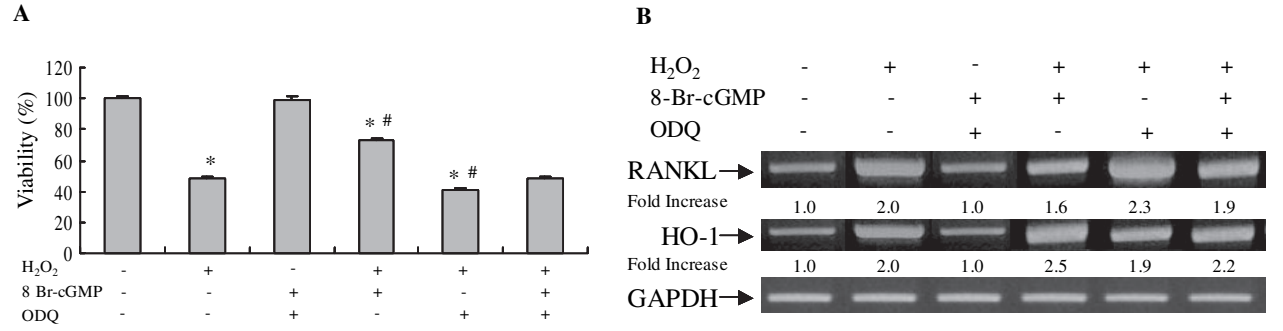

Fig. 3. Effects of the cGMP analog, 8-bromo-cGMP, and an inhibitor of guanylate cyclase (1H-[1,2,4]oxadiazole[4,3-α]quinoxalin-1-one) on H_2O_2-mediated cytotoxicity (A), and on receptor activator of nuclear factor-κB ligand (RANKL) and heme oxygenase-1 expression (B). Cells were treated for 24 h with the indicated concentrations of H_2O_2 (1.0 mM), 8-bromo-cGMP (50 μM), and/or 1H-[1,2,4]oxadiazole[4,3-α]quinoxalin-1-one (100 μM) in periodontal ligament cells. Pretreatment with 8-bromo-cGMP and 1H-[1,2,4]oxadiazole[4,3-α]quinoxalin-1-one was for 12 h. *Statistically significant difference compared with the control group, $p < 0.05$. Statistically significant difference compared with the H_2O_2 group, $p < 0.05$. The same procedure as described in the legend to Fig. 1 was performed. Results are representative of three independent experiments. 8-Br-cGMP, 8-bromo-cGMP; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; ODQ, 1H-[1,2,4]oxadiazole[4,3-α]quinoxalin-1-one.

oxygenase-1 is responsible for the cytoprotective and anti-osteoclastogenic effects of heme oxygenase-1 against H_2O_2, we examined the effects of the CO-releasing molecule, tricarbonyldichlororuthenium(II) dimer (CO-RM, $[Ru(CO)_3(Cl)_2]_2$), and iron on H_2O_2 cytotoxicity in periodontal ligament cells. As shown in Fig. 4A, pretreatment with 10 μM CO-RM effectively blocked RANKL mRNA expression and the cytotoxicity induced by H_2O_2, suggesting that CO may mediate the inhibitory effect of heme oxygenase-1 on H_2O_2 cytotoxicity. In contrast, pretreatment of cells with 0.5 mM Fe^{3+} enhanced both the cytotoxic and RANKL-inducing effects of H_2O_2 (Fig. 1B), indicating that the intracellular iron in periodontal ligament cells is not specific for pathways of the cytotoxicity and RANKL pathway induced by H_2O_2.

Effects of MAP kinase inhibition on the cytotoxic and RANKL mRNA-inducing effects of H_2O_2 in periodontal ligament cells

To examine whether activation of p38 or extracellular signal-related kinase (ERK)1/2 mediates the effects of H_2O_2 in periodontal ligament cells, we next investigated the effects of pharmacological agents that modulate MAP kinase activities. periodontal ligament cells were treated for 24 h with 1.0 mM H_2O_2 and a selective inhibitor for ERK (PD98059), p38 (SB203580) or JNK (JNK inhibitor 1) (Fig. 5). Periodontal ligament cells treated with either 20 μM SB203580 or 20 μM PD98059 blocked the effects of H_2O_2 on cell viability and expression of RANKL and heme oxygenase-1 mRNA, indicating that both p38 MAP kinase and ERK1/2 contribute to the cytotoxic and RANKL mRNA-inducing effects of H_2O_2 in human periodontal ligament cells.

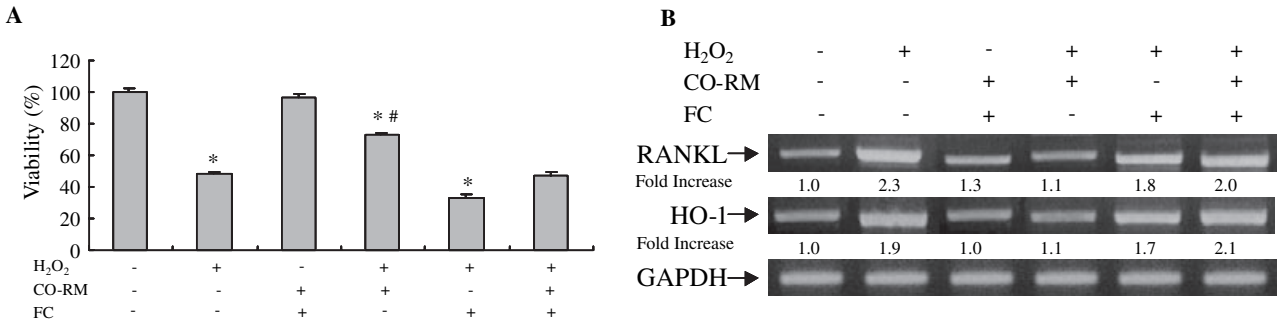

Fig. 4. Effects of carbon monoxide and iron on H_2O_2-mediated cytotoxicity (A), receptor activator of nuclear factor-κB ligand (RANKL) and heme oxygenase-1 expression (B). Cells were treated for 24 h with the indicated concentrations of H_2O_2 (1.0 mM), CO-releasing molecule tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3(Cl)_2]_2$) (10 μM), ferric chloride (Fe^{3+}, 0.5 mM), or combinations thereof, in periodontal ligament cells. Pretreatment with CO-releasing molecule tricarbonyldichlororuthenium(II) dimer or ferric chloride was for 12 h. *Statistically significant difference compared with the control group, $p < 0.05$. Statistically significant difference compared with the H_2O_2 group, $p < 0.05$. The same procedure, as described in the legend to Fig. 1, was performed. Results are representative of three independent experiments. CO-RM, CO-releasing molecule tricarbonyldichlororuthenium(II) dimer; FC, ferric chloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1.

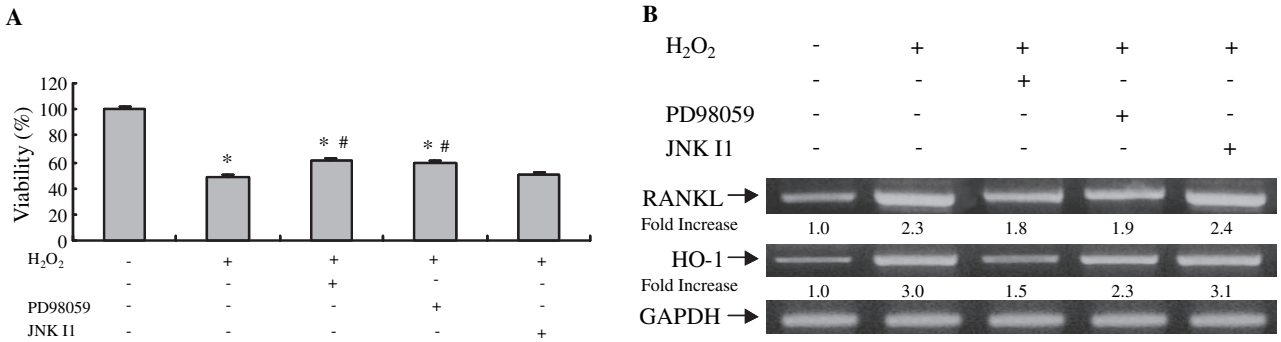

Fig. 5. Effects of mitogen-activated protein (MAP) kinase inhibition on H_2O_2-mediated cytotoxicity (A), receptor activator of nuclear factor-κB ligand (RANKL) and heme oxygenase-1 expression (B). Cells were treated for 24 h with the indicated concentrations of H_2O_2 (1.0 mM), a selective inhibitor for p38 (SB203580; 20 μM), a selective inhibitor for extracellular signal-regulated kinase (PD98059; 20 μM), and JNK inhibitor 1 (20 μM) in periodontal ligament cells. Pretreatment with MAP kinase inhibitors was for 1 h. *Statistically significant difference compared with the control group, $p < 0.05$. Statistically significant difference compared with the H_2O_2 group, $p < 0.05$. The same procedure as described in the legend to Fig. 1 was performed. The results are representative of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; JNK I1, JNK inhibitor 1.

Effects of cycloheximide, pyrrolidine dithiocarbamate, and protein kinase G inhibitor on the cytotoxic and RANKL mRNA-inducing effects of H_2O_2

To investigate whether up-regulation of RANKL mRNA expression by H_2O_2 is dependent on de novo protein synthesis, we pretreated periodontal ligament cell cultures with cycloheximide. Cycloheximide reduced both the cytotoxic and the RANKL and heme oxygenase-1 mRNA-inducing effects of H_2O_2 (Fig. 6).

We next examined the impact of pyrrolidine dithiocarbamate, an antioxidant and an inhibitor of nuclear factor-κB. Pyrrolidine dithiocarbamate reduced the cytotoxic and RANKL mRNA induced by H_2O_2, whereas it increased H_2O_2-induced heme oxygenase-1 mRNA expression. Furthermore, as shown in Fig. 3, the guanylate cyclase inhibitor ODQ counteracts the action of RANKL and heme oxygenase-1, suggesting that the effect of H_2O_2 is mediated by activation of protein kinase G. Indeed, the cytotoxic effect of H_2O_2, as well as its effect on RANKL and heme oxygenase-1 mRNA induction, was blocked by KT5823, a highly specific inhibitor of protein kinase G.

Discussion

The effects of reactive oxygen species on bone-related differentiation may be mediated in two ways: the suppression of bone formation and the stimulation of bone resorption. A recent study by Mody *et al.* (30) showed that reactive oxygen species, including H_2O_2 or superoxide anion, can inhibit osteoblastic differentiation of mouse and rabbit bone marrow stromal cells and calvarial osteoblasts by ERKs and nuclear factor-κB (30,31). Although many reports have shown that reactive oxygen species stimulate osteoclast differentiation and bone

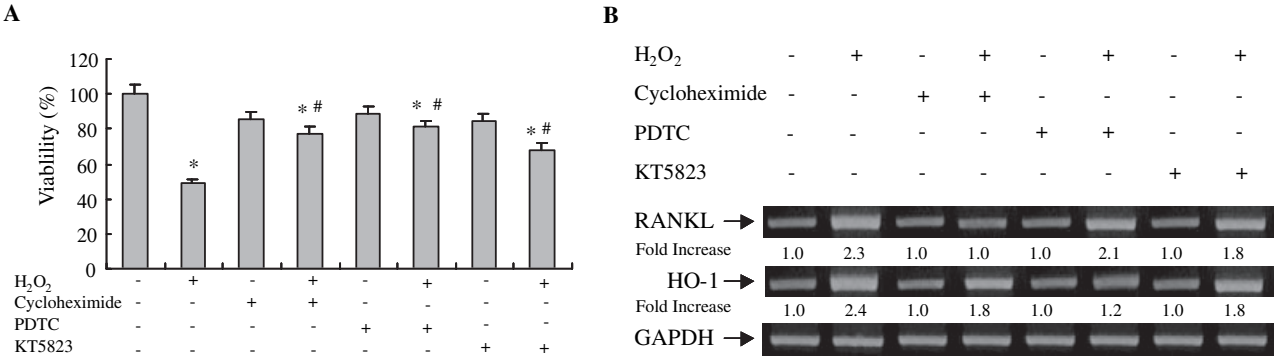

Fig. 6. Effects of various inhibitors of signal transduction on H_2O_2-mediated cytotoxicity (A), receptor activator of nuclear factor-κB ligand (RANKL) and heme oxygenase-1 expression (B). Cells were treated for 24 h with the indicated concentrations of H_2O_2 (1.0 mM), cychloheximide (10 mM), KT5823 (2 mM), and pyrrolidine dithiocarbamate (50 mM) in periodontal ligament cells. Pretreatment with inhibitors was for 1 h. *Statistically significant difference compared with the control group, Statistically significant difference compared with the H_2O_2 group, $p < 0.05$. The same procedure as described in the legend to Fig. 1 was performed. Results are representative of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; PDTC, pyrrolidine dithiocarbamate.

resorption (3–7), little is known about the mechanisms underlying the effects of reactive oxygen species in periodontal ligament cells, which actively regulate the formation and resorption of alveolar bone.

The present study demonstrates that H_2O_2 significantly reduces the viability of human periodontal ligament cells, and that heme oxygenase-1 is associated with cytoprotection against H_2O_2 in these cells. These results are consistent with the results of other studies on the cytotoxic and heme oxygenase-1-inducing effects of H_2O_2 (22–26). H_2O_2 was cytotoxic to periodontal ligament cells, and it increased RANKL mRNA expression in these cells in a concentration- and time-dependent manner (Fig. 1A–D); however, in periodontal ligament cells exposed to H_2O_2, expression of osteoprotegerin was stable, suggesting that osteoclast differentiation under H_2O_2 stress is mediated independently of osteoprotegerin in periodontal ligament cells.

The mechanisms by which heme oxygenase-1 inhibit osteoclastogenesis and protect cells from oxidant stress have not been fully clarified. In the present study, we noted that exposure of cells to H_2O_2 after hemin pretreatment increased heme oxygenase-1 mRNA expression and decreased RANKL mRNA expression, as compared with H_2O_2 exposure alone (Fig. 2B). Twenty-four hours of exposure to hemin greatly reduced the cytotoxicity of 1 mM H_2O_2 (Fig. 2A), whereas exposure to zinc protoporphyrin IX increased it. Thus, H_2O_2 cytotoxicity may be correlated with expression of RANKL and heme oxygenase-1 mRNAs in a reciprocal manner.

In many biological systems, H_2O_2 targets the enzyme soluble guanylate cyclase, which produces cGMP. In addition, cGMP has been reported to participate in H_2O_2-dependent heme oxygenase-1 induction and in the protective function of heme oxygenase-1 in smooth muscle cells and astrocytes (27,32). When we examined the regulatory role of cGMP using the membrane-permeable cGMP analogue, 8-bromo-cGMP, we found that it inhibited H_2O_2-induced cytotoxicity, up-regulated the heme oxygenase-1 mRNA level, and down-regulated the RANKL mRNA level. Furthermore, we found that the soluble guanylate cyclase inhibitor, ODQ, decreased induction of heme oxygenase-1 expression and increased induction of RANKL mRNA by H_2O_2 (Fig. 3B). Thus, 8-bromo-cGMP and ODQ had contrasting effects on H_2O_2 cytotoxicity and H_2O_2-induced RANKL mRNA expression; 8-bromo-cGMP attenuated these responses and ODQ enhanced them (Fig. 3A). These results provide evidence that the cGMP signaling system is part of the heme oxygenase-1-associated protective response against H_2O_2 in human periodontal ligament cells.

The end products of heme breakdown by heme oxygenase (CO, free iron and bilirubin) have been shown to reduce the cytotoxicity of H_2O_2, peroxynitrite, and serum deprivation (33–35). The cytoprotective effect of heme oxygenase-1 has recently been associated with increased cellular iron efflux through the up-regulation of an iron pump that remains to be fully characterized (33). Furthermore, heme oxygenase-1-mediated bilirubin production has been reported to reduce the toxicity of cyclosporin A in neuronal cells (36). Although CO is generally believed to play an important role in reducing oxidative injury, experimental results appear contradictory. CO may have a cytoprotective effect against hypoxic lung injury (37), but Rothfuss *et al.* have reported that CO is not involved in the antioxidative action of heme oxygenase-1 in lymphocytes (38). In the present study, CO, but not ferric chloride, effectively reduced the cytotoxic and RANKL mRNA-inducing effects of H_2O_2 in periodontal ligament cells (Fig. 4), suggesting that CO plays a role in the cytoprotective effect of heme oxygenase-1 induction.

Much evidence suggests that H_2O_2 activates the MAP kinase family of serine/threonine kinases, which includes ERKs, p44/42 MAP kinase,

c-jun NH_2-terminal kinase (JNK), and p38 kinase (39,40). Some studies have shown that the H_2O_2-mediated barrier function in microvascular endothelial cells is regulated by p38 MAP kinase, but not by ERK or JNK (41), whereas other studies have suggested that ERK1/ERK2 phosphorylation leads to increases in endothelial solute permeability in response to H_2O_2 (42). In our study, treatment of periodontal ligament cells with H_2O_2 in the presence of the specific inhibitors of p38 (SB203580) or ERK (PD98059) decreased H_2O_2 cytotoxicity and H_2O_2-induced expression of heme oxygenase-1 and RANKL mRNAs, suggesting that ERK1/ERK2 and p38 are involved in the cytotoxic and osteoclast differentiation effects of H_2O_2.

Previous studies have shown that de novo synthesis of mRNA and protein is required for induction of apoptosis by oxidant stimuli (43,44). Here, we demonstrated that cycloheximide significantly reduces H_2O_2 cytotoxicity and its inductive effect on RANKL mRNA expression, suggesting that de novo protein synthesis-dependent pathways may mediate the expression of RANKL mRNA expression in periodontal ligament cells.

Many of the cellular effects of cGMP are mediated by protein kinase G (32). In our study, the protein kinase G inhibitor, KT5823, blocked the cytotoxic and RANKL/heme oxygenase-1 mRNA-inducing effects of H_2O_2. Therefore, these findings suggest that cGMP analogs block H_2O_2 cytotoxicity in periodontal ligament cells by activating protein kinase G, although the exact mechanism by which cGMP/protein kinase G prevents cytotoxicity and osteoclastogenesis is not known.

Nuclear factor-κB activation leads to the transcription of genes encoding signaling and defense proteins. Recently, pyrrolidine dithiocarbamate, one of the most effective inhibitors of nuclear factor-κB, was found potentially to activate expression of endogenous antioxidant genes, such as heme oxygenase-1 (44,45 46), which is consistent with our finding that pyrrolidine dithiocarbamate up-regulates heme oxygenase-1 gene expression. Moreover, pyrrolidine dithiocarbamate blocked the cytotoxic and RANKL mRNA-inducing effects of H_2O_2 in our study, suggesting that activation of nuclear factor-κB in periodontal ligament cells may result in the expression of proteins required for bone resorption, as well as being involved in protection against oxidative stress-induced damage.

It has been suggested that parathyroid hormone or transforming growth factor-β stimulates RANKL expression via the cAMP-dependent kinase/cAMP response element binding protein (PKA/CREB) pathway and that CREB may be a central regulator of RANKL expression in mouse stromal/osteoblast cells (47). Because reactive oxygen species also stimulate PKA-CREB signaling in PC12 cells (48), the effect of PKA/CREB signaling on H_2O_2-mediated regulation of heme oxygenase-1 or the RANKL pathway in human cells remains to be discovered.

In summary, H_2O_2 has direct cytotoxic effect on human periodontal ligament cells, which sequentially induces the defense protein, heme oxygenase-1, and up-regulates RANKL mRNA. Heme oxygenase-1 has a cytoprotective function that involves activation of soluble guanylate cyclase and the cGMP pathway, and may be mediated through generation of CO and up-regulation of cellular iron efflux. Furthermore, it appears that MAP kinases, protein kinase G, and nuclear factor-κB play significant roles in mediating the cytotoxic and osteoclast differentiation effects of H_2O_2. Therefore, although another pathway may exist for the protective activity of heme oxygenase-1 against the cytotoxic and osteoclastogenic effects of H_2O_2, we conclude that heme oxygenase-1 appears to be a principal defense molecule against the osteoclast differentiation and cytotoxic effects of H_2O_2 in human periodontal ligament cells.

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