Original Article

The role of heme oxygenase-1 in mechanical stress- and lipopolysaccharide-induced osteogenic differentiation in human periodontal ligament cells

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ABSTRACT

Objective: To investigate the mechanisms through which mechanical stress and lipopolysaccharide treatment modulate osteoblastic differentiation in periodontal ligament cells.

Materials and Methods: Cells were treated with lipopolysaccharide and/or mechanical strain applied with a Flexercell Strain Unit. Protein expression and mRNA were analyzed by Western blotting and reverse transcription–polymerase chain reaction, respectively.

Results: When lipopolysaccharide was co-applied with mechanical strain, the increase in the expression of bone morphogenetic protein-2, bone morphogenetic protein-7, and Runx2 mRNA seen with mechanical strain alone was restricted, but heme oxygenase-1 expression was further enhanced. Furthermore, pretreatment with an inhibitor of heme oxygenase-1 or inhibitors of p38, mitogen-activated protein kinase, JNK, phosphoinositide 3-kinases, protein kinase G, and nuclear factor κB restricted osteogenic differentiation induced by the application of lipopolysaccharide and mechanical strain.

Conclusions: These results suggest that orthodontic force-induced osteogenesis in alveolar bone is inhibited by the accompanying periodontal inflammation via the upregulation of heme oxygenase-1 expression. Thus, the heme oxygenase-1 pathway could provide a possible therapeutic strategy to improve bone formation in orthodontic treatment. (*Angle Orthod.* 2010;80:740–747.)

KEY WORDS: Mechanical stress; LPS; Osteogenic differentiation; HO-1; PDLCs

INTRODUCTION

Lipopolysaccharide (LPS), a main component of the cell surface of Gram-negative bacteria, is thought to be a major cause of inflammation by bacterial infections. A previous study demonstrated that *Porphyromonas gingivalis* and *Escherichia coli* LPS exhibit a high affinity for orthodontic brackets.[1] This affinity can affect the concentration of LPS in the gingival sulcus, thereby contributing to inflammation in periodontal tissues adjacent to the brackets.[2] In a dog model, orthodontically treated teeth showed additional loss of connective tissue attachment in sites with infrabony pockets and plaque-induced inflammation.[3] However, the effects of the combination of orthodontic force and oral bacterial infection on bone remodeling are not completely understood.

The transmission of orthodontic force to the alveolar bone is mediated by the response of the periodontal ligament cells (PDLCs).[4] The cells in the periodontal ligament are capable of differentiating into osteoblasts or cementoblasts in response to a mechanical stimulus.[5,6] The osteogenic differentiation of PDLCs is known to play a pivotal role in alveolar bone remodeling during orthodontic tooth movement.[4]

In the bone microenvironment, bone morphogenetic proteins (BMP) play a critical role in the regulation of osteoblast differentiation and function. BMP-2, BMP-4, BMP-6 and BMP-7 are growth factors in promoting the

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differentiation of mesenchymal stem cells (MSCs) into osteoblasts or chondroblasts.[7] Moreover, BMP activity is regulated by antagonists such as noggin.[8] In human osteoblastic cells, mechanical stress (MS) stimulates mineralization by increasing the production of BMPs and decreasing the production of BMP antagonists.[9]

Mechanical signals may promote cell differentiation into a particular phenotype.[10] For example, MS has been shown differentiate MSCs into the ligament lineage but not into bone or cartilage,[11] PDLCs into osteoclasts,[12] MSCs into chondrocytes,[13] MSCs into bone,[14] and dental pulp stem cells into dentin.[15] However, MS such as cyclic tension has been reported to both inhibit[16] and stimulate[17] the synthesis of alkaline phosphatase. In mice, orthodontic force-induced osteoclastogenesis in alveolar bone was inhibited by the accompanying periodontal inflammation and resulted in reduced orthodontic tooth movement.[18] But there is no evidence to show that MS and LPS affect the induction of growth factor genes, specifically BMPs, in PDLCs.

Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme-generating biliverdin. Previously, we reported that the HO-1 pathway is a key mechanism for the adaptation to stressful conditions and the recovery from injurious events by dental cells.[19–24] Moreover, the expression of HO-1 is related to adipogenesis by human MSCs,[25] osteoblastic differentiation by PDLCs[23] and neuronal differentiation by MSCs.[26]

Because HO-1 is associated with differentiation, it is plausible to postulate that HO-1 is involved in the response of PDLCs to MS and bacterial infection as well as in the differentiation of PDLCs to osteoblast-like cells. The objective of this study was to examine the effects of MS and LPS on the osteodifferentiation of PDLCs. We also examined the underlying signaling pathways, by measuring changes in the expression of HO-1, BMPs, BMP antagonists, and transcription factor involved in osteoblastic differentiation.

MATERIALS AND METHODS

Cell Culture

Human immortalized PDLCs were established from normal PDLCs through HPV16 *E6* and *E7* genes transfection.[27] The PDLCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, Md) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C. For differentiation, PDLCs were cultured with differentiation medium (10% FBS/DMEM, including 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate), as described previously.[28] This study was approved by the institutional review board and ethical committee at Wonkwang University.

Application of MS

Human PDLCs (3×10^5/well) were subcultured into six-well, 35-mm flexible-bottomed Uniflex culture plates (Flexcell Corp, Hillsborough, NC) with a centrally located rectangular portion (15.25 mm $\times$ 24.18 mm) coated with type I collagen designed to provide a uniform uniaxial strain. They were then subjected to an intermittent deformation of 3%, 6%, 12% or 15% of maximum stretch for 2.5 seconds followed by 2.5 seconds of relaxation (12 cycle/min for 24 h) with a Flexercell FX-4000 Strain Unit (Flexcell Corporation, Hillsborough, NC) according to the manufacturer's instructions.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

After applying the MS, total RNA was isolated from the cells using the Trizol reagent (Invitrogen Life Technologies, Carlsbad, Calif) according to the manufacturer's instructions. Briefly, 1 μg of RNA isolated from each cultures was reverse transcribed using oligo (dT)[15] primers (Roche Diagnostics, Mannheim, Germany) and AccuPower RT PreMix (Bioneer, Daejon, Korea) according to the manufacturer's protocols. An amount of cDNA equivalent to 25 ng of total RNA was then subjected to polymerase chain reaction (PCR). The primers used for cDNA amplification are listed in Table 1. PCR products were subjected to electrophoresis on 1.2% agarose gel and were stained with ethidium bromide.

Western Blot Analysis

An equal volume of 2 $\times$ sodium dodecyl sulfate (SDS) sample buffer was added and the samples were then boiled for 5 minutes. A sample (40 μg) was subjected to electrophoresis on 12% SDS-polyacrylamide gels for 2 hours at 20 mA and then transferred onto nitrocellulose. The membrane was incubated for 1 hour in 5% (wt/vol) dried milk protein in phosphate buffer solution (PBS) containing 0.05% (vol/vol) Tween-20 (PBS-T), washed in PBS-T and then incubated for 1 hour in the presence of primary antibody (1:1,000). The membrane was washed extensively with PBS-T and then incubated with anti-mouse IgG antibody conjugated to horseradish peroxidase (1:3,000) for 1 hour. After extensive washes, immunoreactive bands on the membrane were visualized using chemiluminescent reagents according to the manufacturer's protocol (Amersham-Pharmacia, Piscataway, NJ).

Table 1. Reverse Transcription–Polymerase Chain Reaction Primers

Gene	Sequence (5′ -3′)	Size (bp)
Heme oxygenase-1	Forward: AAGATTGCCCAGAAAGCCCTGGAC Reverse: AACTGTGCCACCAGAAAGCTGAG	405
Bone morphogenetic protein-2	Forward: CCAACCATGGATTCGTGGTG Reverse: GGTACAGCATCGAGATAGCA	456
Bone morphogenetic protein-7	Forward: CAGCCTGCAAGATAGCCATT Reverse: AATCGGATCTCTTCCTGCTC	276
Runx2	Forward: AACCCACGAATGCACTATCCA Reverse: CGGACATACCGAGGGACCTG	75
Noggin	Forward: GCACCCAGCGACAACCTGCCC Reverse: GCTGCCCACCTTCACGTAGCG	399
Glyceraldehyde 3-phosphate dehydrogenase	Forward: ACCACAGTCCATGCCATCAC Reverse: TCCACCACCCTGTT GCTGTA	452

Statistical Analysis

The statistical analyses of the data were performed by one-way analysis of variance followed by a multiple-comparison Turkey's test with the use of the SPSS program (SPSS 12.0, SPSS GmbH, Munich, Germany). Statistical significance was determined at $P < .05$.

RESULTS

The Effects of MS on Osteodifferentiation in PDLCs

In PDLCs, MS increased the levels of BMP-2, BMP-7, and Runx2 mRNAs in a force-dependent and time-dependent manner (Figure 1A,B). In contrast, MS resulted in a force-dependent and time-dependent decrease in noggin mRNA. The maximal expression of BMP-2, BMP-7, and Runx2 mRNAs was observed in cells subjected to MS applied at a force causing a 12% increase in cell length for 48 hours.

The Effects of MS on HO-1 Expression in PDLCs

As shown in Figure 1A,B, HO-1 mRNA expression gradually increased in PDLCs treated with MS. This time-dependent and force-dependent upregulation peaked at 12% of MS and decreased with greater force. Along with this upregulation in HO-1 mRNA expression, we also found a corresponding increase in HO-1 protein expression (Figure 1C,D).

Effects of MS and LPS on Osteogenic Differentiation and HO-1 Expression in Human PDLCs

To examine the combined effect of LPS and MS on osteogenic differentiation in human PDLCs, we applied a force resulting in 12% cellular elongation with LPS derived from *P gingivalis* for 48 hours, and then reverse transcription–PCR was performed. LPS decreased MS-induction of BMP-2, BMP-7, and Runx-2, a key transcription factor associated with osteoblast differentiation in PDLCs. However, 0.1 μg/mL and 1 μg/mL LPS enhanced MS-induced HO-1 upregulation and noggin downregulation (Figure 2).

Effects of Inducing or Inhibiting HO-1 Expression on MS-induced and LPS-induced Osteogenic Differentiation

To verify the role of HO-1 in counteracting the osteoblastic differentiation caused by LPS and MS in PDLCs, we investigated the effect of pretreating cells with protoporphyrin IX chloride (CoPP, a potent HO-1 inducer) or tin-protoporphyrin IX (SnPP, a potent HO-1 inhibitor) for 16 hours. CoPP pretreatment increased while SnPP blocked the induction of HO-1 mRNA expression by LPS treatment and MS. The LPS-induced and MS-induced BMP-2, BMP-7, and Runx-2 mRNA upregulation was enhanced by CoPP pretreatment (Figure 3). Also, pretreatment with CoPP and the corresponding rise in HO-1 prevented the downregulation of noggin mRNA seen with LPS and MS. In contrast, SnPP pretreatment prevented the LPS-induced and MS-induced osteogenic differentiation. SnPP also inhibited HO-1 expression, and this inhibition enhanced the decrease in noggin mRNA expression by exposure to LPS and MS.

Effects of Signal Transduction Modulators on MS-induced and LPS-induced Osteogenic Differentiation

To examine the signaling pathways involved in MS-induced and LPS-induced osteogenic differentiation and HO-1 expression, PDLCs were pretreated with various inhibitors of key signaling molecules. MS-induced and LPS-induced osteogenic differentiation and HO-1 expression were inhibited in PDLCs by the selective p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, the JNK inhibitor SP600125, the specific membrane-permeable proteasome inhibitor MG132, the phosphoinositide 3-kinases (PI3K) inhibitor LY294002, the protein kinase G (PKG)

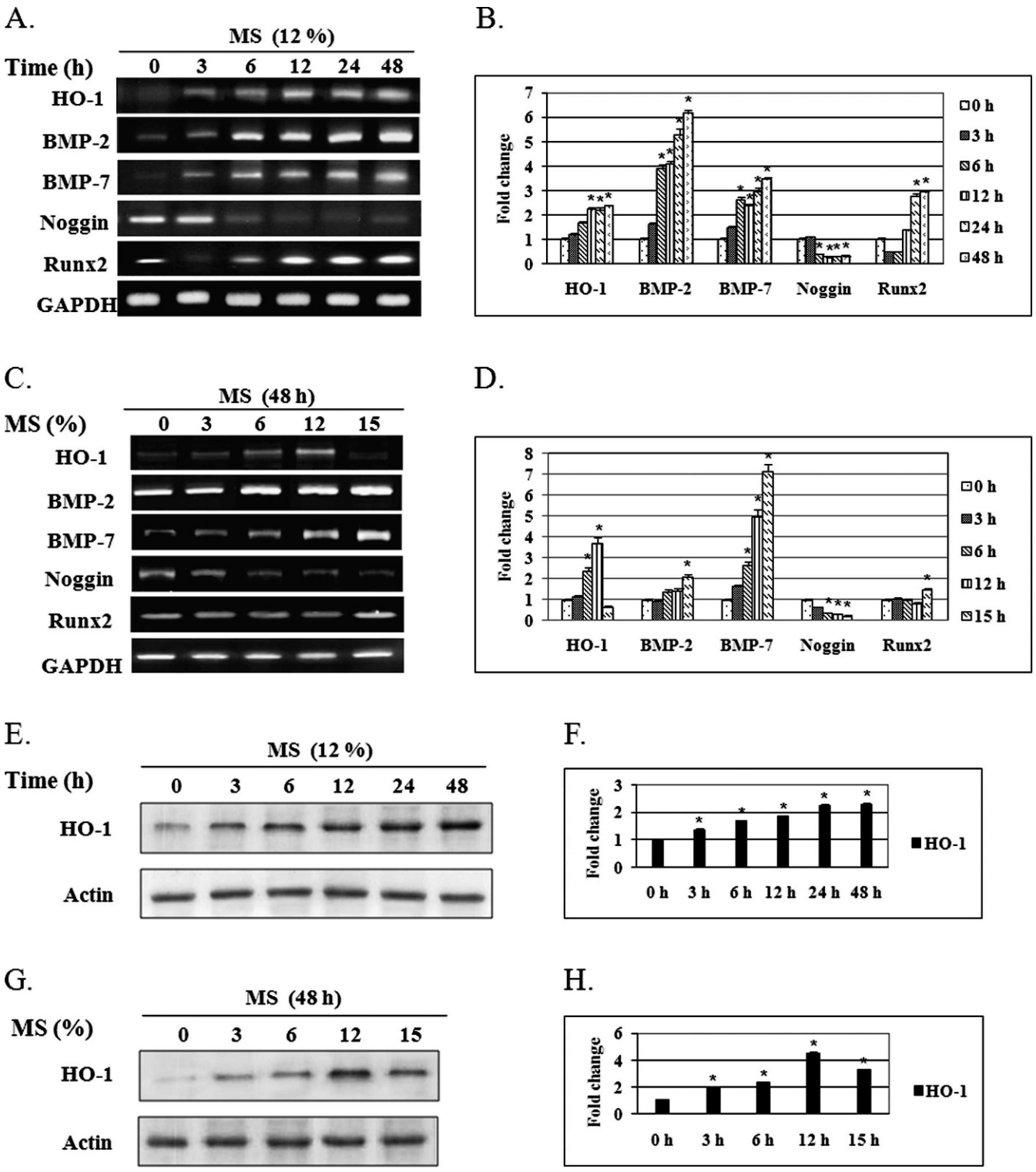

Figure 1. Effects of mechanical stress on expression of heme oxygenase-1 and osteogenic differentiation markers in periodontal ligament cells. Cells were cultured with or without mechanical stress (3%–15%) for up to 48 hours. mRNA and protein were assayed by semiquantitative reverse transcription–polymerase chain reaction (A and C) and Western blotting (E and G). Quantitative data on the relative amounts of mRNA or protein of genes to glyceraldehyde 3-phosphate dehydrogenase or β-actin are provided (B, D, E, and F). Experiments were performed in triplicate for each data point, and the standard errors are shown as error bars. *Statistically significant difference compared with control, $P < .05$.

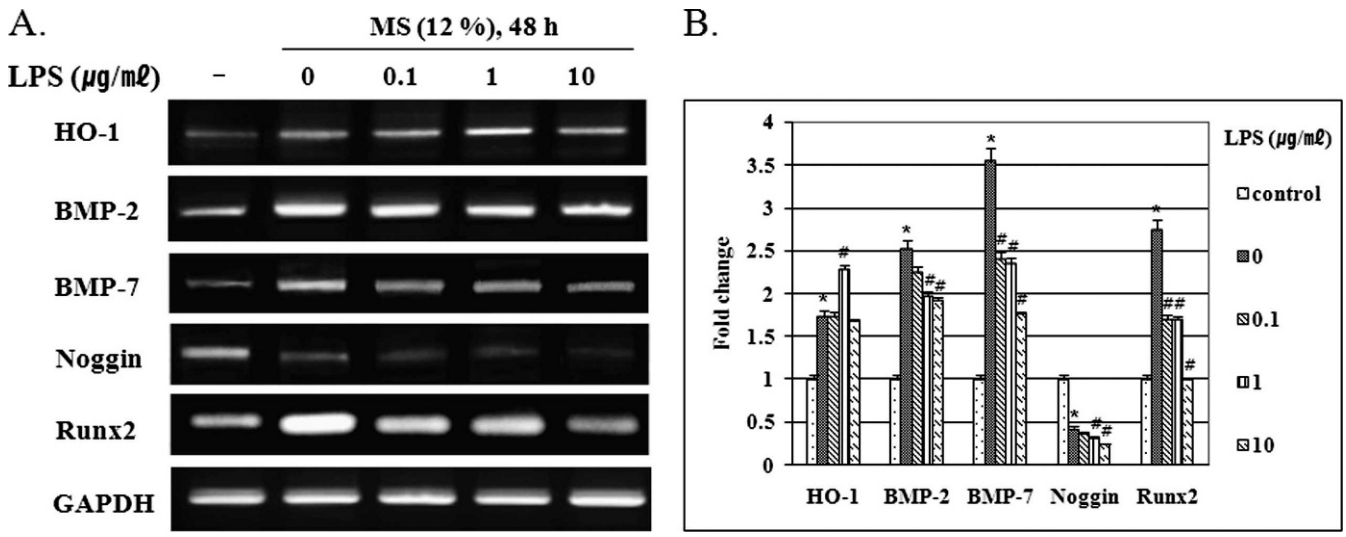

Figure 2. Effects of mechanical stress (MS) and lipopolysaccharide from *Porphyromonas gingivalis* on osteogenic differentiation and heme oxygenase-1 expression. Cells cultured with or without indicated concentrations of lipopolysaccharide and MS (12%) for 48 hours were assayed by reverse transcription–polymerase chain reaction (A). Quantitative data on the relative amounts of mRNA genes to glyceraldehyde 3-phosphate dehydrogenase are provided on the right (B). These data are representative of three independent experiments. *Statistically significant difference compared with control, $P < .05$; #statistically significant difference compared with MS-treated group, $P < .05$.

inhibitor KT5823, and the nuclear factor κB (NF-κB) inhibitor PDTC. Osteogenic differentiation and HO-1 expression were not inhibited by the ERK1/2 pathway inhibitor PD98059 (Figure 4).

DISCUSSION

Human PDLCs have been widely used to study the effects of tensile MS on the protein and mRNA expression of inflammatory cytokines,[29] cytoskeletal components,[16] and osteogenic genes.[17,30,31] In this study, we provide evidence for a role of the HO-1 pathway and relevant signaling molecules in the regulation of LPS-induced and strain-induced osteoblastic differentiation.

BMPs are known to play a critical role in proliferation, differentiation, and matrix secretion of bone cells.[31] In the present study, we found that MS on

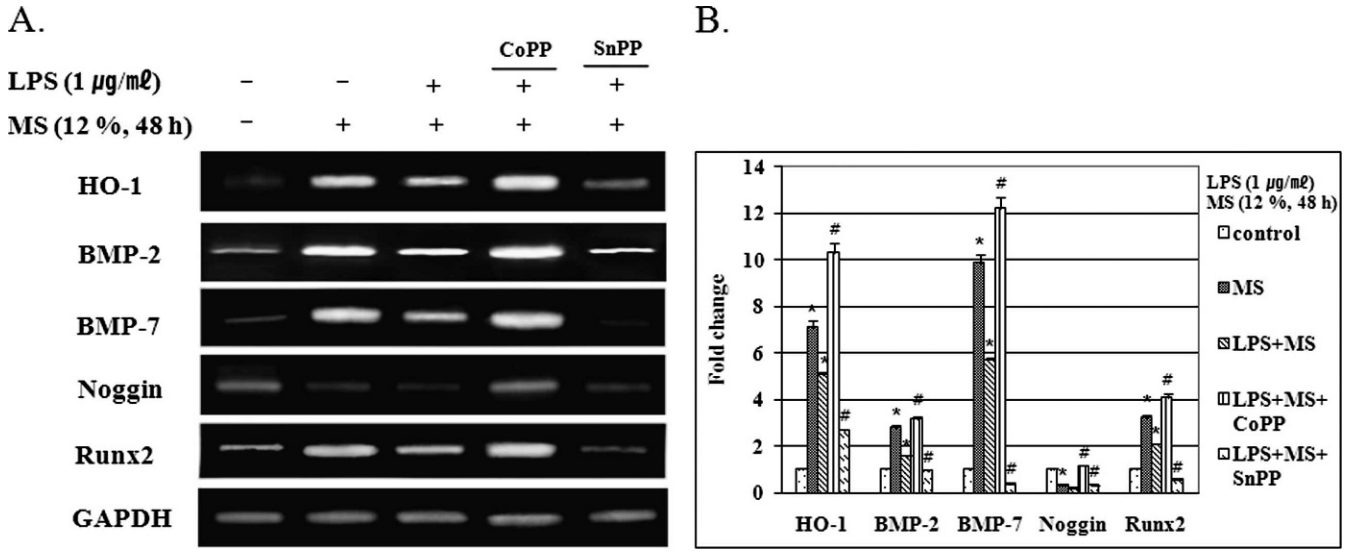

Figure 3. Effects of heme oxygenase-1 (HO-1) inducer and inhibitor on osteogenic differentiation induced by mechanical stress and lipopolysaccharide (LPS). Cells were pretreated with different concentrations of tin-protoporphyrin IX (an HO-1 inhibitor) or protoporphyrin IX chloride (an HO-1 inducer) for 16 hours and then stimulated with or without strain and LPS for an additional 48 hours (A). Quantitative data on the relative amounts of mRNA genes to glyceraldehyde 3-phosphate dehydrogenase are provided on the right (B). *Statistically significant difference compared with control, $P < .05$; #statistically significant difference compared with group treated with mechanical stress and LPS, $P < .05$. The experiments were performed three times, and representative data are shown.

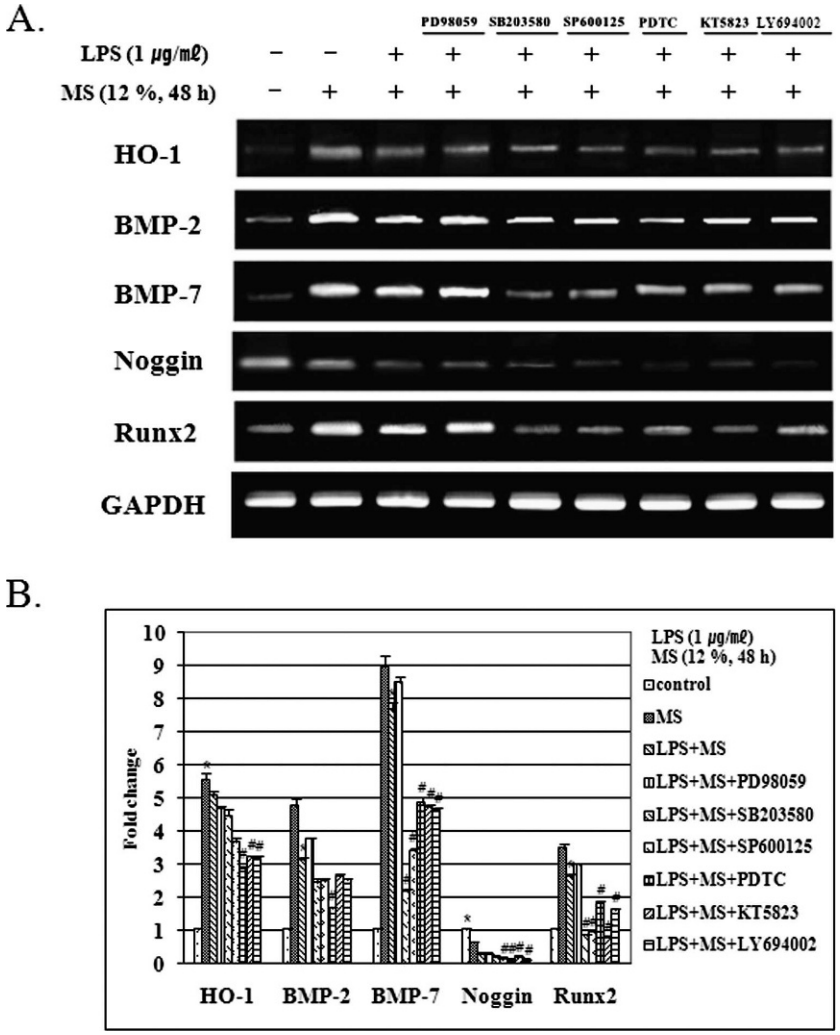

Figure 4. Effects of signal transduction modulators on osteogenic differentiation induced by mechanical stress and lipopolysaccharide (LPS). Cells were pretreated with 20 μM SB203580, 20 μM PD098059, 20 μM SP600125, 10 μM PDTC, 20 μM KT5823, and 20 μM LY694002 for 1 hour and then stimulated with or without strain and LPS for an additional 48 hours. Quantitative data on the relative amounts of mRNA genes to glyceraldehyde 3-phosphate dehydrogenase are provided (B). The experiments were performed three times, and representative data are shown. *Statistically significant difference compared with control, $P < .05$; #statistically significant difference compared with group treated with mechanical stress and LPS, $P < .05$.

PDLCs induced the transcriptional expression of BMP-2 and BMP-7. These data, therefore, are in agreement with previous studies showing that MS upregulates BMP-2, BMP-4, BMP-6, and BMP-7 in osteoblasts[14]; BMP-2, BMP-6, and BMP-7 in osteoblasts[32]; and BMP-2 and BMP-6 in PDLCs.[30]

There is evidence for the autoregulation of BMP expression in osteoblasts in the form of a negative feedback loop that decreases cellular exposure to BMPs. Thus, in this study, we focused on extracellular antagonists of BMP, such as noggin. We found that the expression of BMP antagonists decreased with the application of MS in a force-dependent manner. From these findings, mechanical loading may stimulate osteoblastic differentiation by regulating the expression of BMPs and BMP antagonists.

It has been well documented that BMPs upregulate various transcription factors involved in osteoblastic differentiation.[14,33] Thus, we determined the effect of MS on the expression of Runx2 transcription factor. Our results showed that the expression of Runx2 mRNA was significantly increased by the application of MS, which suggests that MS may promote osteoblastic differentiation through the regulation of BMP-responsive transcription factors in PDLCs.

HO-1 is emerging as the prototypic endogenous cytoprotective enzyme essential for cells to adapt to stressful conditions and to recover from injurious events.[19–24] In the present study, we found that MS increased HO-1 mRNA and protein expression in PDLCs. These findings are consistent with our previous study in which osteogenic differentiation

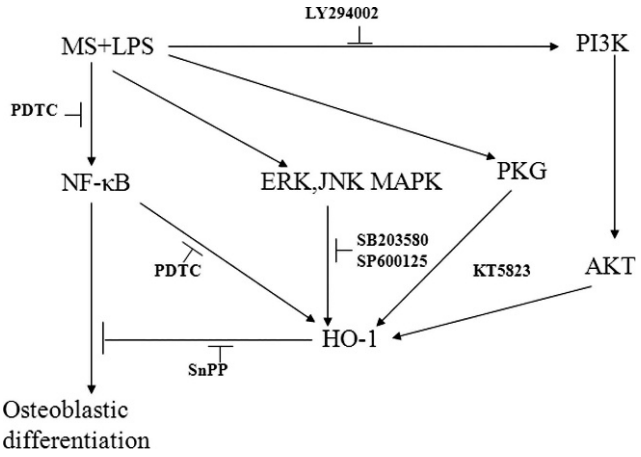

Figure 5. Schematic diagram illustrating the signaling pathways triggered by exposure to mechanical stress and lipopolysaccharide in periodontal ligament cells.

was found to be associated with HO-1 expression in PDLCs.[24] Thus, upregulation of HO-1 may provide a major contribution to cellular adaptation and resistance to MS.

It is not known whether LPS and MS affect osteoblastic differentiation, such as BMP expression in PDLCs. In this study we found that LPS potently inhibited MS-induced BMP-2, BMP-7, and Runx2 expression, whereas LPS recovered noggin expression. These results imply that LPS acts as a negative modulator of MS-induced osteoblastic differentiation.

Antioxidant and cytoprotective as well as anti-inflammatory and immunosuppressive effects have been described following HO-1 induction by CoPP.[34] Thus, the effect of pharmacologic HO-1 induction was tested using CoPP. When applied in our PDLCs, the HO-1 inducer CoPP enhanced the LPS-induced and MS-induced osteoblastic differentiation. This CoPP-enhanced differentiation was abrogated by SnPP treatment, proving the specificity of this HO-1 effect. Thus, for the first time, we have demonstrated the effects of HO-1 induction on MS-induced and LPS-induced osteogenic differentiation in PDLCs.

HO-1 gene expression can be induced via signaling pathways involving MAPKs (ERK, JNK, p38), PI3K, and NF-κB in PDLCs.[21,22,24] Increases in the expression of HO-1, BMP-2, BMP-7, and Runx-2 resulting from treatment with LPS and MS were attenuated by preincubation with selective inhibitors of p38, ERK, PI3K, PKG, and NF-κB (SB203580, U0126, LY294002, KT5823, and PDTC, respectively). These results suggest that LPS-induced and MS-induced HO-1 and BMP expression occurs via the activation of p38, ERK MAPK, PI3K, PKG, and NF-κB. Figure 5 is a schematic representation of the signaling pathway involved in HO-1 and BMP activation in response to LPS and MS in PDLCs.

CONCLUSIONS

- To our knowledge this study is the first to demonstrate that MS-induced osteogenic differentiation of PDLCs is inhibited by the accompanying periodontal inflammation.
- HO-1 may influence changes on the differentiation in PDLCs.

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