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Roles of MAP Kinases in the Regulation of Bone Matrix Gene Expressions in Human Osteoblasts by Oscillatory Fluid Flow

Chia-Ching Wu,[1,2] Yi-Shuan Li,[2] Jason H. Haga,[2] Nanping Wang,[2] Ian Y-Z. Lian,[2] Fong-Chin Su,[1] Shunichi Usami,[2] and Shu Chien[2]*

[1]Institute of Biomedical Engineering, National Cheng Kung University, Tainan, Taiwan
[2]Department of Bioengineering and Whitaker Institute of Biomedical Engineering, University of California San Diego, California 92093

Abstract We investigated the effects of oscillatory flow in regulating the gene expressions of type I collagen (COL1, the main component of human bone tissues) and osteopontin (OPN, the key gene for calcium deposition) in human osteoblast-like (MG-63) cells, and the roles of mitogen-activated protein kinases (MAPKs) in this regulation. The cells were subjected to oscillatory flow (0.5 ± 4 dyn/cm^2) or kept under static condition for various time periods (15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 16 h). Oscillatory flow caused significant up-regulations of both COL1 and OPN gene expressions over the 16 h of study, and a transient activation of MAPKs was starting at 15 min and declining to basal level in 2 h. The flow-induction of COL1 was blocked by an ERK inhibitor (PD98059) and reduced by a JNK inhibitor (SP600125), whereas that of OPN was abolished by PD98059. Analysis of the cis-elements in the COL1 and OPN promoters suggests the involvement of transacting factors Elk-1 and AP-1 in the transcription regulation. The ERK inhibitor (PD98059) blocked Elk-1 phosphorylation, as well as COL1 and OPN gene expression. The JNK inhibitor (SP600125) abolished c-jun phosphorylation and COL1 expression. These results suggest that the flow-induction of OPN was mediated through the ERK-Elk1-OPN pathway, and that COL1 was regulated by both the ERK-Elk1-COL1 and JNK-c-JUN-COL1 pathway. J. Cell. Biochem. 98: 632–641, 2006. © 2006 Wiley-Liss, Inc.

Key words: collagen; osteopontin; gene expression; human bone matrix; MAPK; osteoblast; shear stress; signal transduction

The human bone tissues provide mechanical supports for weight bearing and limb movements. Mechanical stimulations due to fluid flow, matrix strain, and loading-induced electric fields have potent effects on bone cell metabolism [Turner, 1998], including the modulations of bone density, strength, adaptation, and fatigue at the macroscopic level and also cellular remodeling, molecular signaling, and gene expression at the microscopic and sub-microscopic levels [Stein et al., 1996; Boskey et al., 1999].

There are three types of cells in the human bone tissue: osteoblasts, osteocytes, and osteoclasts. The osteoblasts serve the functions of synthesizing bone extracellular matrix (ECM), regulating the calcium deposition and mineralization, and responding to mechanical stimuli. The osteocytes regulate the maintenance of osteoid matrix and blood-calcium homeostasis. The osteoclasts, which have a polarized shape with multinucleated cells, are the regulators of bone resorption [Salgado et al., 2004].

There is evidence that mechanical forces-induced signal transduction can be initiated by increasing the intracellular calcium concentration [Hung et al., 1996; Chen et al., 2000] as a

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*Correspondence to: Shu Chien, MD, PhD, Departments of Bioengineering and Medicine, Whitaker Institute of Biomedical Bioengineering, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093—0412. E-mail: shuchien@ucsd.edu

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result of opening the stretch-activated (SA) channel [Naruse et al., 1998], and the consequent release of nitric oxide (NO) [Johnson et al., 1996; Turner et al., 1997; McAllister and Frangos, 1999; Bakker et al., 2001]. Recent studies also demonstrated that mechanotransduction in bone cells involves the sequential activations (via phosphorylation cascade) of various intracellular signaling molecules, including mitogen-activated protein kinases (MAPKs) [Kushida et al., 2001; You et al., 2001; Jessop et al., 2002; Weyts et al., 2002], phosphoinositide 3-kinase (PI3k)/Akt [Danciu et al., 2003], and protein kinases B and C [Biggs et al., 1999; Geng et al., 2001]. As a result, mechanical signals can activate transcription factors such as activator protein-1 (AP-1) [Peverali et al., 2001], bone-specific transcriptional regulator (Cbfa1) [Franceschi, 1999; Ziros et al., 2002], and NF-κB [Granet et al., 2001] to modulate the expression of genes that regulate different physiological functions.

It has been hypothesized that the normal blood pressure provides a baseline level of flow in the lacunar/canalicular network in bone structure in vivo to maintain bone metabolism [Hillsley and Frangos, 1994] and that changes in mechanical loading cause alterations in intraosseous pressure and fluid flow along the pressure gradients. These mechanical loadings in vivo due to daily living events, for example, locomotion, usually are dynamic and oscillatory in nature [Hung et al., 1996]. Flow stimulation has been shown to induce prostaglandin production [Klein-Nulend et al., 1995; Ajubi et al., 1996; Reich et al., 1997], alkaline phosphatase secretion [Hillsley and Frangos, 1997], NO release [Johnson et al., 1996], and calcium activation [Hung et al., 1996; Jacobs et al., 1998] in osteoblast-like cells. However, the signal transduction pathways that lead to the modulation of expression of bone matrix genes, for example, collagen, by oscillatory fluid flow stimuli still remain unclear.

The processes of bone remodeling/maintenance are controlled by the regulation of type I collagen (COL1) and osteopontin (OPN). The synthesis of ECM in bone cells plays an important role in bone remodeling. The expression of COL1, the main ECM component of bone tissues, can be induced by mechanical cyclic stretch in osteoblasts [Wang et al., 2003]. COL1 synthesized in the ECM can serve functions such as providing the appropriate elastic properties for bone tissues to sustain tensile loading. OPN is a non-collagenous, glycosylated phosphoprotein associated with biomineralization in osseous tissues. Calcification plays a major role in maintaining the rigidity of human bone tissues for support and locomotion. The expression of OPN in MC3T3-E1 osteoblasts has been shown to be regulated by oscillatory fluid flow [You et al., 2001]. Both COL1 and OPN are essential for the regulation of bone matrix and the modulation of functionality and mechanical properties. A deficiency in either COL1 (which is important for ECM synthesis) or OPN (which regulates calcium deposition) can lead to severe dysfunctions in human bone tissues. Therefore, we investigated the effects of oscillatory fluid flow on the expression levels of both COL1 and OPN by quantification with real-time PCR. In order to elucidate the signaling pathways involved in the flow-induced regulation of COL1 and OPN expressions in bone cells, we determined (a) the activations of ERK1/2, JNK1/2, and p38 in response to oscillatory flow, and (b) the effects of specific inhibitors for these MAPKs on shear-induced COL1 and OPN expressions. The results have allowed the assessment of the roles of each specific MAPK in the gene expression of COL1 and OPN with/without oscillatory flow conditions.

MATERIALS AND METHODS

Cell Culture and Inhibitor Treatments

MG-63 cells were cultured to confluency on glass slides in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL), 1% penicillin–streptomycin, and maintained in a humidified incubator at 37°C with 5% CO_2.

The pharmaceutical inhibitors PD 098059 (10 μM, CalBiochem, CA), SP600125 (10 μM, CalBiochem), and SB 203580 (10 μM, Biomol, CA) were used to block the activities of MAPK kinase (MEK), JNK, and p38, respectively. Before fluid flow experiments, the cells were incubated with DMEM containing 0.5% FBS for 24 h followed by the treatments with these blocking reagents for 30 min to inhibit the specific kinase functions. The inhibitors were included in the flow system while the cells were subjected to the flow stimulations.

Oscillatory Flow Stimulation

The cells were subjected to oscillatory fluid flow in a system previously described [Usami et al., 1993; Hillsley and Frangos, 1997]. In brief, the flow chamber was created by sandwiching a silicon gasket between an acrylic base and a cell-seeded glass slide. The total area that is subjected to shearing is 2.5 cm wide and 5.0 cm long, and with a height of 0.025 cm (Fig. 1). The flow in the narrow gap between the two plates is laminar with a parabolic velocity profile. The wall shear stress on the surface of the plate (τ_{wall}) can be calculated as $\tau_{wall} = \Delta P(h/2L) = 6Q\mu/Wh^2$, where ΔP is the pressure difference between the inlet and the outlet of the flow channel, h, L, W and are the width, length, and gap height of the rectangular flow channel, respectively, Q is the volume flow rate, and μ is the absolute fluid viscosity.

The cells in the flow chamber were subjected to oscillatory flow or kept under static condition for various time periods (15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 16 h). The oscillatory flow is composed of (a) a low level of mean flow (shear stress = 0.5 dyn/cm^2) supplied by the hydrostatic flow system to provide the basic nutrient and oxygen delivery, and (b) the superimposition of a sinusoidal oscillation using a piston pump with a frequency of 1 Hz and a peak-to-peak amplitude of ± 4 dyn/cm^2. The pressure readings were verified by using pressure transducers (Fig. 1). The parameters of the oscillatory flow were chosen on the basis of fluid flow loading estimated for osteocytes in the canaliculi [Weinbaum et al., 1994]. The pH of the medium was maintained constant by gassing with a mixture of 5% CO_2 and 95% air, and the temperature was maintained at 37°C by using a feedback control system.

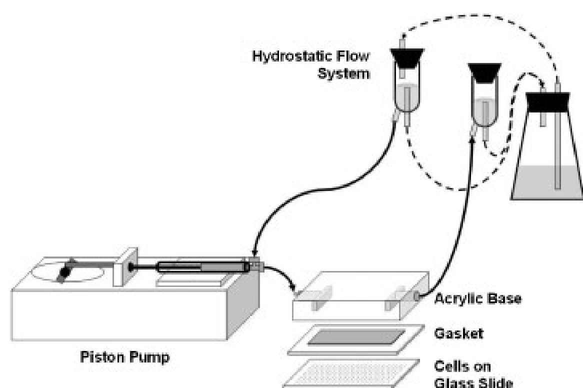

Fig. 1. A schematic drawing of the oscillatory flow system. Oscillatory flow (with a sinusoidal waveform and peaks of ± 4 dyn/cm^2) was created by the pistol pump located in the path of flow application to the cells (solid line). The basic nutrient and oxygen delivery was supplied with a continuous low level of flow (at 0.5 dyn/cm^2) that was controlled by the difference of hydrostatic pressure between two reservoirs. The overflow and new supply of medium was circulated between the two reservoirs (dash line).

Real-Time PCR

Total RNA was isolated from cells that had been subjected to different durations of shear flow and from static control cells by extraction with Trizol as suggested by the manufacturer (Life Technologies, Inc.). Five micrograms of total RNA were used for first-strand cDNA synthesis by using SuperScript™ II Reverse Transcriptase (Invitrogen, Inc.) with Oligo(dT)$_{12-18}$ (Invitrogen, Inc.). After reverse transcription, 1 µg of cDNA was used as a template for amplification in Real-time PCR (MyiQ single color Real-time PCR detection system, Bio-Rad Laboratories). The fluorescent signals produced by COL1- and OPN-specific oligonucleotide probes were quantified during each cycle of the PCR primer extension.

The human type I collagen alpha 1 (COL1A1) primer was designed from the data base of NCBI (Accession: NM_000088) and Primer Express software with forward and reverse sequences (forward: 5′-GCGGCTCCCCATTTTTATACC-3′; reverse: 5′-GCTCTCCTCCCATGTTAAA-TAGCAC-3′). The human OPN (secreted phosphoprotein 1) primer was designed using the sequence data from NCBI (Accession: NM_000582) and modified from Wang-Rodriguez et al. [2003]. The forward and reverse sequences of the OPN primer pairs are 5′-TTGCAGCCTTCTCAGCCAA-3′ and 5′-GG-AGGCAAAAGCAAATCACTG-3′, respectively. The house-keeping gene GAPDH was used as a reference. The primer concentrations for the reference and target genes in the final PCR reaction were optimized, and agarose gel electrophoretic analyses were performed to confirm the specificity of PCR products and to check for the presence of primer–dimer formations in the absence of a template.

The relative gene expression data were analyzed by using the $2^{-\Delta\Delta C_t}$ method [Livak and Schmittgen, 2001]. The experimental results were normalized by using the endogenous reference gene GAPDH, and the ratios to

the untreated control provided the data for folds of change in gene expression.

Immunoblotting

After the experiments, the cells were washed twice with cold phosphate-buffered saline (PBS) and harvested on ice. The cells were then lysed by using the lysis buffer that was slightly modified from Akagi et al. [2002] and contained: 10 mM Tris-HCl, 150 mM NaCl, 2.5 mM EDTA, 10% glycerol, 1% Igepal/NP-40, 50 mM NaF, 20 mM Na pyrophosphate, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 2 mM Na_3VO_4, 1 mM PMSF. Cell lysate supernatants containing 20 µg of total proteins were transferred to nitrocellulose membranes following sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on 10% crosslinked gels. The membranes were blocked by 5% BSA in TBS with 0.1% Tween-20 for 90 min.

The activations of MAPKs were detected by immunoblotting, using antibodies (PD98059, SP600125, and SB203580) that specifically recognize the phosphorylated forms of ERK1/2 (1:2,000, Cell Signal Technology), JNK1/2 (1:1,000, Promega), and p38 (1:1,000, Cell Signal Technology), respectively. PD98059 blocks ERK1/2 phosphorylation by acting on MEK1 [Alessi et al., 1995], and SP600125 blocks JNK1/2 phosphorylation by acting on both c-jun N-terminal kinase and MKK4 [Vaishnav et al., 2003]. SB203580 selectively inhibits p38 kinase activity, that is, the phosphorylation of MAPKAPK-2 (MAPK activated proteinkinase-2) by p38 [Taniyama et al., 2004]. Antibody of the phosphorylated form of MAPKAPK-2 (1:2,000, Cell Signal Technology) was used to assess the degree of inhibition of p38 kinase activity after treatment with SB203580. The phosphorylated forms of c-jun (1:1,000, Santa Cruz) and Elk-1 (1:1,000, Santa Cruz) antibodies were used to assess the activations of MAPK downstream targets involved in flow-induced bone matrix gene regulation. The polyclonal anti-ERK2 (1:4,000, Santa Cruz), anti-JNK1 (1:1,000, Santa Cruz), anti-p38 (1:1,000, Santa Cruz), anti-c-jun/AP1 (1:1,000, Santa Cruz), and β-actin (1:1,000, Sigma) were used to indicate the amounts of protein loading. The bound primary antibodies were detected using appropriate secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL detection system (Amersham, Uppsala, Sweden).

Statistical Analysis

Data reported are means ± standard errors from three independent experiments for each time point and each treatment group. The differences between the control and treatment groups were analyzed by one-way ANOVA and Bonferroni test for multiple comparisons, with P-value <0.05 taken as statistically significant.

RESULTS

Both COL1 and OPN Were Up-Regulated by Oscillatory Flow

The COL1 mRNA level in MG-63 cells was quantitatively measured by real-time PCR as a function of shearing time (Fig. 2A). Oscillatory flow caused a significant up-regulation of COL1 mRNA between 4 and 16 h. There was no significant difference in COL1 expression levels between 4 and 16 h, with an average of about 2.5-fold of the static control. OPN expression was elevated after 30 min of oscillatory flow and increased progressively with continued flow stimulation, reaching a maximum level of 7.27 ± 1.57 fold at 16 h (Fig. 2B).

Temporal Differences in MAPK Activations

To investigate the effects of oscillatory flow on the mechanotransduction of MAPKs, MG-63 cells were subjected to oscillatory flow or kept in static condition for 0, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 16 h. The activations of the major MAPKs were determined from their phosphorylation levels by immunoblotting with anti-phospho-ERK1/2, anti-phospho-JNK1/2, and anti-phospho-p38 (Fig. 3A).

Oscillatory flow caused transient activations of all three MAPKs between 15 min and 1 h, and the activities returned to control levels at 2 h. The peak values (folds) at 30 min were 2.72 ± 0.54 for ERK1, 4.30 ± 0.60 for ERK2, 4.26 ± 0.76 for JNK1, 1.84 ± 0.31 for JNK2, and 1.44 ± 0.23 for p38 (Fig. 3B).

Having studied the temporal effects of oscillatory flows on the expression of COL1 and OPN and on the activities of MAPKs, we proceeded to investigate the effects of inhibiting each of the three MAPKs on their activation status in MG-63 cells under static and oscillatory flow conditions.

In static controls, PD98059 and SP600125 had no significant effects on the basal phosphorylation status of ERK1/2 and JNK1/2, respectively,

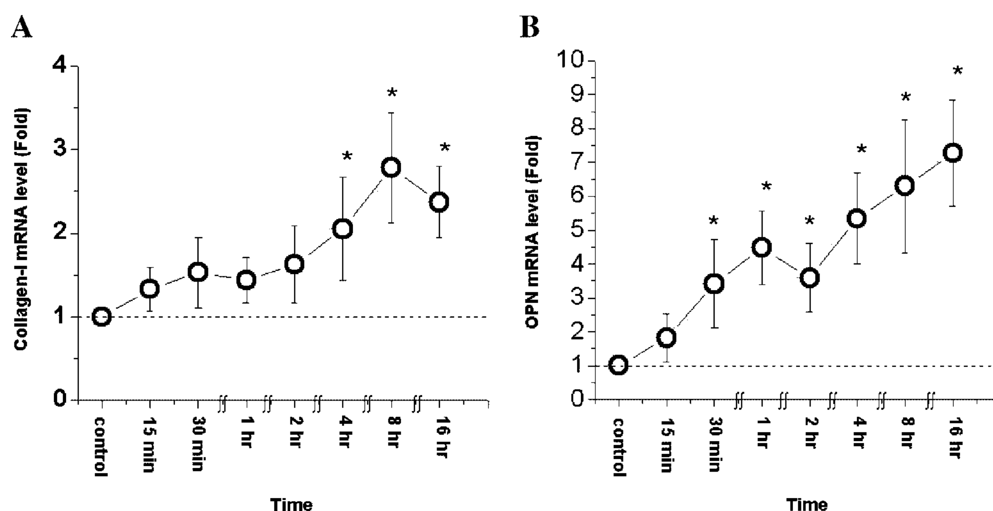

Fig. 2. Time courses of expression levels of COL1 (**A**) and OPN (**B**) mRNA under oscillatory flow. RNA samples were isolated from human MG-63 osteoblast-like cells exposed to oscillatory flow for the indicated time periods. After reverse transcription, the samples were subjected to real time PCR using specific primer sets for COL1 (A) and OPN (B). *: significant difference from control (no flow) with $P < 0.05$.

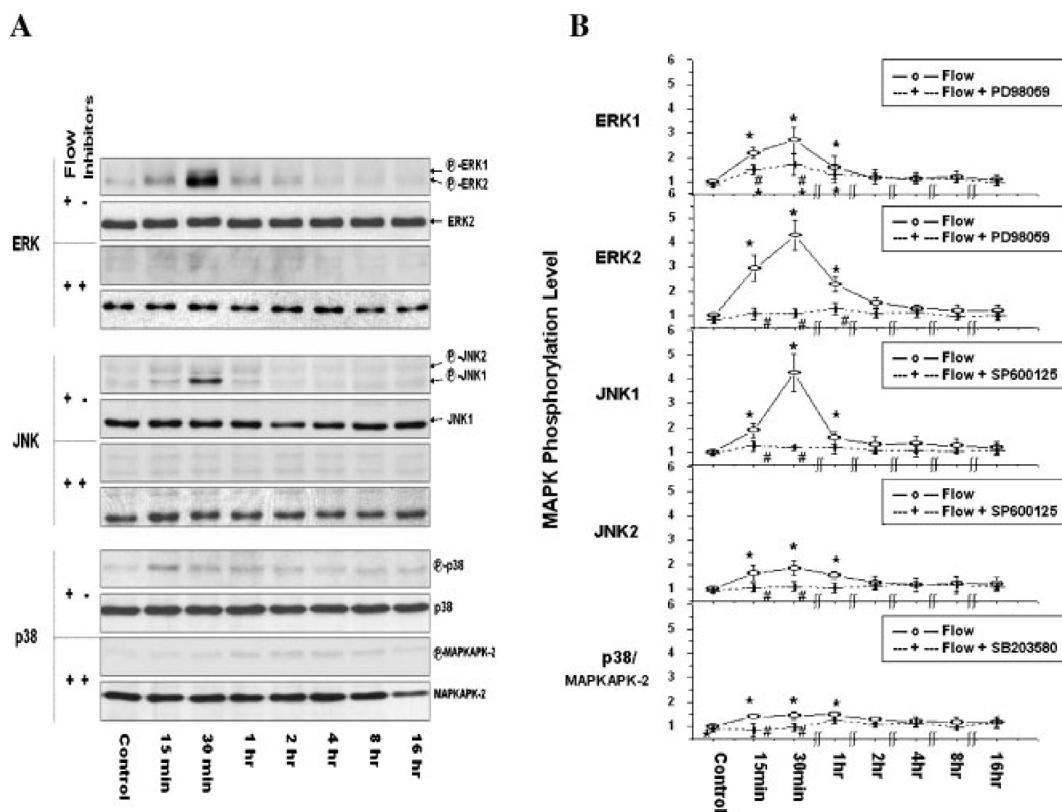

Fig. 3. Phosphorylation levels for the three major types of MAPKs (ERK1/2, JNK1/2, and p38) in MG-63 cells. Measurements were made under static condition (control) or oscillatory flows for the time periods as indicated, and also following treatments with the inhibitors PD098059 (10 μM), SP600125 (10 μM), and SB203580 (10 μM). **A**: The Western blot results of the phosphorylation of MAPKs. **B**: Plots of densitometric results of MAPK phosphorylation (mean ± standard errors) against time. *: significant difference ($P < 0.05$) from control (no flow). #: significant difference ($P < 0.05$) between inhibitor treatment and the corresponding untreated control under the same flow condition.

whereas SB203580 suppressed p38 kinase activity slightly to 0.8-fold (Fig. 2A). Under oscillatory flow, PD98059 significantly reduced (but did not abolish) the flow-induced ERK1 activation and it totally abolished the flow-induced ERK2 activation. SP600125 (JNK inhibitor) totally abolished the flow-induced activations of both JNK1 and JNK2 (Fig. 3B). SB203580 (p38 inhibitor) reduced the flow-induced MAPKAPK-2 phosphorylation. These results indicate that the inhibitors used exerted inhibitory effects on the flow-activation of each of their cognate MAPKs.

Up-Regulation of COL1 by Oscillatory Flow Is Mediated by Both ERK and JNK, and the Up-Regulation of OPN by Oscillatory Flow Is Mediated by ERK

MG-63 cells were subjected to oscillatory flows for 8 h in the presence of the inhibitors PD98059, SP600125, or SB203580 to assess their effects on the flow-induced COL1 and OPN gene expression. The time of 8 h was chosen because this is when the COL1 expression reached a maximum under oscillatory flow.

Under oscillatory flow, the inhibition of ERKs with PD98059 not only blocked the up-regulation of COL1, but actually decreased the expression to below the static control level ($P < 0.05$) (Fig. 4A). Inhibition of JNK (SP600125) significantly reduced the increase of COL1. The p38 inhibitor (SB203580) did not have significant blocking effects on the oscillatory flow-induced COL1 expression. These results indicate that the up-regulation of COL1 by oscillatory flow is mediated through the ERK and JNK pathways. The oscillatory flow-induced up-regulation of OPN expression was significantly attenuated by the inhibition of ERKs but not by the inhibitions of JNK and p38. These results indicate that the up-regulation of OPN expression by oscillatory flow is mediated by ERKs (Fig. 4B).

We further examined the cis-elements in the promoters of COL1 [Ghosh, 2002] and OPN [El-Tanani et al., 2004] to identify the potential trans-acting factors that serve as the targets for ERK and JNK activations. Among the potential factors, we chose to study Elk-1 and c-JUN because they have been shown to be involved in the COL1 and OPN expression regulated by TGF-β, TNF-α, and other activated steroid hormone receptors [Sodek et al., 1995; McGaha et al., 2002; Verrecchia et al., 2002]. In our current study, oscillatory flow significantly induced the phosphorylation levels of both Elk-1 and c-JUN (Fig. 5). SP600125, which abolished the flow-induced phosphorylation of JNK, also inhibited the phosphorylation of transcription factor c-jun, but did not affect the phosphorylation of Elk-1, suggesting that c-JUN may be involved in the JNK-mediation of flow-induced COL1 expression (Fig. 5). On the other hand, the flow-induced phosphorylation of Elk1 was abolished after treated with PD98059, suggesting that Elk-1 may be involved in the

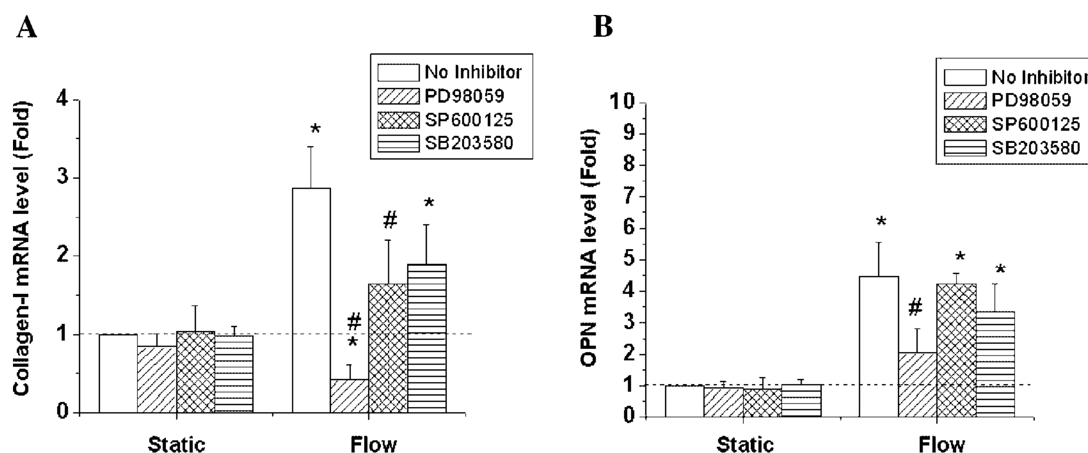

Fig. 4. Effects of inhibitors of ERK, JNK, and p38 on the expression levels of COL1 (**A**) and OPN (**B**) under static condition or oscillatory flow. Cells were exposed to oscillatory flow (or kept under static condition) with or without the presence of PD98059 (10 μM), SP600125 (10 μM), or SB203580 (10 μM). RNA samples were isolated and subjected to real time PCR to determine the expression levels of COL1 and OPN. *: significant difference ($P < 0.05$) from static control without treatment. #: significant difference ($P < 0.05$) between inhibitor treatment and the corresponding untreated control under the same flow condition.

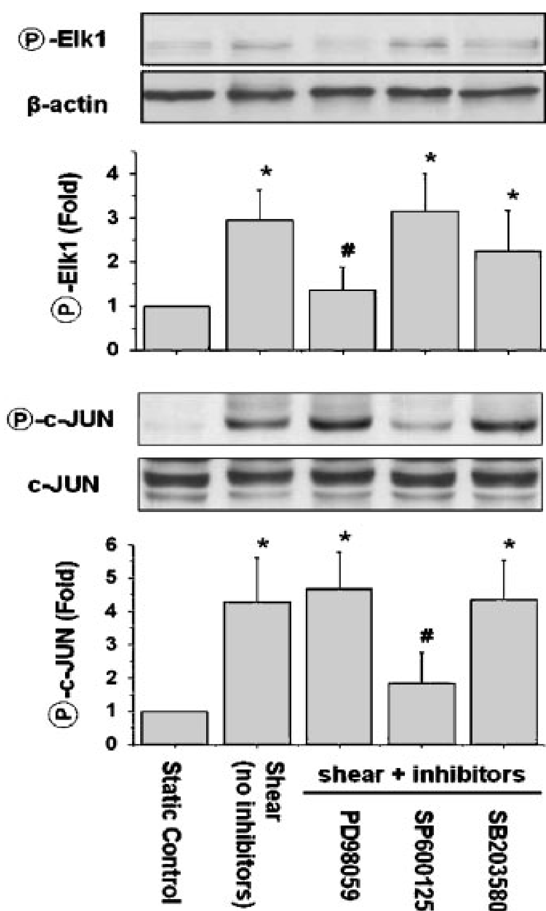

Fig. 5. Effects of PD98059 and SP600125 on flow-induced Elk-1 and c-JUN phosphorylation. Oscillatory flow significantly induced Elk1 phosphorylation. The flow induction of Elk-1 phosphorylation was abolished by PD98059, but not affected by SP600125. Oscillatory flow also induced significant phosphorylation of c-JUN, which was attenuated by SP600125, but not by PD98059. *: significant difference ($P < 0.05$) from static control without treatment. #: significant difference ($P < 0.05$) between inhibitor treatment and the corresponding untreated control under the same flow condition.

ERK mediation of flow-induced COL1and OPN expressions (Fig. 5).

DISCUSSION

To the best of our knowledge, this is the first study to show that oscillatory flow induces the expression of COL1 through the MAPKs-dependent signal transduction pathway. The results on the mRNA levels of COL1 and OPN provide some insights into the regulation of ECM in human bone tissue by oscillatory flow. Both collagen and OPN are essential elements in the processes of bone remodeling/maintenance. A deficiency in either COL1 or OPN can lead to severe dysfunctions in human bone tissues. Defects in the production of collagen, such as osteogenesis imperfecta, result in fragile bones, while the calcification function is still normal [Termine et al., 1984]. It was shown that repetitive mechanical stretching of smooth muscle cells caused an enhancement of collagen production as revealed by the quantitation of the hydroxyproline and proline concentrations for collagen and noncollagen proteins [Sumpio et al., 1988]. Lee et al. [2004] demonstrated that mechanical tensile forces stimulated COL1 synthesis at the mRNA level in anterior cruciate ligament fibroblasts. In the dentoalveolar tissue of rats subjected to experimental tooth movement for 1 day, in situ hybridization showed a high level of COL1 mRNA expression in the osteoblasts on the tension side [Domon et al., 2001].

To test the effect of short-term (30-min) flow on the regulation of OPN and COL1 expression, we subjected the cells to oscillatory flow (0.5 ± 4 dyn/cm^2) for 30 min followed by changing the flow to the low mean flow without oscillation (0.5 dyn/cm^2, for nutrition supply) for various total time periods (1, 2, 4, 8, 16 h). The 30-min short-duration of oscillatory flow was chosen based on the peak MAPKs phosphorylation time point under flow (Fig. 3), as well as its relevance to the duration of normal mechanical loading for daily activity and exercises. The results showed that the 30-min oscillatory flow caused transient mRNA up-regulation of both COL1 (2–4 h) and OPN (1–4 h). The expressions of both COL1 and OPN declined to basal level after 8 h (data not shown). These results indicate that short-term flow can cause the activation of signaling events and lead to the up-regulation of bone matrix regulation gene in a transient manner, whereas continuous long-term flow leads to a more sustained gene expression (Fig. 2).

Our studies indicate that ERK signaling plays a significant role in the regulation of COL1 and OPN under oscillatory flow. The ERK $\frac{1}{2}$ activation induced by oscillatory flow has later peak values and shorter durations as compare to our previous results under steady flow [Weyts et al., 2002], suggesting that the temporal regulation of mechanotransduction may be flow-pattern dependent. There is evidence that activations of MAPK pathways may be mediated by an increase of intracellular calcium concentration [Hung et al.,

1996; Turner et al., 1997; Naruse et al., 1998; McAllister and Frangos, 1999; Chen et al., 2000; Bakker et al., 2001]. Jessop et al. observed that the flow-induced activation of ERK1 in osteoblast-like cells (ROS 10/2.8 cells) is caused by calcium influx via L-type channels. They also found that the flow-induced activation of ERK is modulated by NO and prostacyclin [Jessop et al., 2002].

Our current study showed that ERK activities can be linked to the flow-induced phosphorylation of Elk1. The Elk1, one of the Ets family transcription factors, is a DNA-binding protein through the helix-turn-helix motif of its Ets domain and activates the transcription as a result of its phosphorylation. The Est binding site plays an essential role in the activation of SP1, which can mediate the COL1 transcription in response to TGF-β stimulation in fibroblasts [Zhang et al., 2000; Czuwara-Ladykowska et al., 2001]. Therefore, the COL1 promoter containing the SP1 sequence (located between the −129 and −107 fragment) is a possible target binding site for the flow-induced up-regulation of COL1 mediated by the ERK-Elk pathway.

With regard to the flow-induced OPN expression, the structure of the OPN promoter contains three consensus TCF-binding sites, three Ets-binding sites, and one AP-1-binding site [El-Tanani et al., 2004]. The TCF-binding sites in the OPN promoter have been shown to promote OPN transcription when bound by the TCF/Elk1 protein activated by ERK phosphorylation [Kortenjann et al., 1994; El-Tanani et al., 2001; Denhardt et al., 2003]. In the present study, the flow-induced OPN transcriptional regulation can be correlated with ERK and Elk phosphorylation and might involve both TCF and Ets binding sites.

The up-regulation of COL1 expression by oscillatory flow also involves JNK1/2 signaling. Our data provide a possible signaling mechanism for flow-induced COL1 expression that involves the JNK-c-JUN pathway. The phosphorylation of c-jun, the major component of the AP-1 transcription complex, may cause the transcription of COL1 via the AP-1 binding site (located between +292 and +67 in COL1 promoter). The AP-1 binding site has been identified as one of the important cis-elements that regulate COL1 expression by Ras-transformed fibroblasts [Slack et al., 1995]. The COL1 promoter contains both SP1 and AP1 binding sites, which have been shown to be responsible for the TGF-β regulation of COL1 production in fibroblasts [Cirillo et al., 1999; Zhang et al., 2000]. In the present study, we show that the flow-induced phosphorylation of MAPKs and their specific downstream proteins may target to different binding sites of COL1 promoters through ERK-Elk1 and JNK-c-JUN pathways.

Flow-dependent activation of JNK is regulated by mechanisms involving β/γ, Ras, and tyrosine kinase(s) in bovine aortic endothelial cells [Li et al., 1996; Jo et al., 1997]. Flow-dependent activation of JNK in human primary osteoblast-like (HOB) cells and human MG-63 bone cells has been shown to be involved with calcium signaling pathways and integrin binding [Peake et al., 2000]. In our study, SP600125

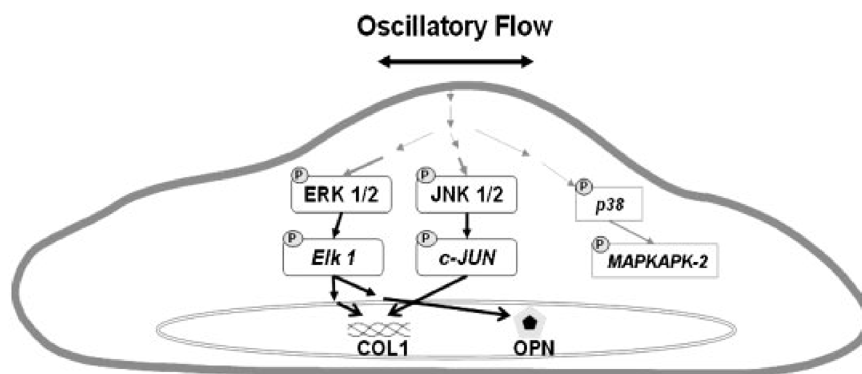

Fig. 6. Schematic summary of the possible molecular mechanisms by which oscillatory flow regulates the expression levels of COL1 and OPN.

caused a complete blocking of flow-induced JNK1/2 phosphorylation and reduced the up-regulation of COL1 under oscillatory flow.

The present approach of determining gene expressions (COL1 and OPN) and MAPK activities with/without inhibitors provides new insights into the molecular bases of the signaling pathways that modulate human bone matrix gene expression. Based on the results described above, the possible COL1 and OPN regulation pathways for bone cells in response to oscillatory flow stimulation can be diagrammatically summarized in Figure 6.

CONCLUSIONS

The results of our studies on human osteoblast-like cells indicate that both COL1 and OPN expressions are up-regulated by oscillatory flow. The use of inhibitors for individual MAPKs revealed that the flow-induction of COL1 expression is mediated by ERK-Elk1 and JNK-c-JUN pathways, and the flow-induction of OPN is mainly through the ERK-Elk1 pathway.

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