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Ras Modulation of Superoxide Activates ERK-dependent Angiogenic Transcription (HIF-1α) and VEGF-A Expression in Shock Wave-Stimulated Osteoblasts*

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Vascular endothelial growth factor (VEGF) released by osteoblasts plays an important role in angiogenesis and endochondral ossification during bone formation. In animal studies, we have reported that shock waves (SW) can promote osteogenic differentiation of mesenchymal stem cells through superoxide-mediated signal transduction (Wang, F. S., Wang, C. J., Sheen-Chen, S. M., Kuo, Y. R., Chen, R. F., and Yang, K. D. (2002) *J. Biol. Chem.* 277, 10931–10937) and vascularization of the bone-tendon junction. Here, we found that SW elevation of VEGF-A expression in human osteoblasts to be mediated by Ras-induced superoxide and ERK-dependent HIF-1α activation. SW treatment (0.16 mJ/mm^2, 1 Hz, 500 impulses) rapidly activated Ras protein (15 min) and Rac1 protein (30 min) and increased superoxide production in 30 min and VEGF mRNA expression in 6 h. Early scavenging of superoxide, but not nitric oxide, peroxide hydrogen, or prostaglandin E$_2$, reduced SW-augmented VEGF-A levels. Inhibition of superoxide production by diphenyliodonium, an NADPH oxidase inhibitor, was found to suppress VEGF-A expression. Transfection of osteoblasts with a dominant negative (S17N) Ras mutant abrogated the SW enhancement of Rac1 activation, superoxide synthesis, and VEGF expression. Further studies demonstrated that SW significantly promoted ERK activation in 1 h and HIF-1α phosphorylation and HIF-1α binding to VEGF promoter in 3 h. In support of the observation that superoxide mediated the SW-induced ERK activation and HIF-1α transactivation, we further demonstrated that scavenging of superoxide by superoxide dismutase and inhibition of ERK activity by PD98059 decreased HIF-1α activation and VEGF-A levels. Moreover, culture medium harvested from SW-treated osteoblasts increased vessel number of chick chorioallantoic membrane. Superoxide dismutase pretreatment and anti-VEGF-A antibody neutralization reduced the promoting effect of conditioned medium on angiogenesis. Taken together, we have showed that SW-induced Ras and Rac1 activation followed by superoxide-mediated ERK activation and HIF-1α activation resulted in an increase of VEGF expression and angiogenesis *in vivo*. Thus, an appropriate modulation of redox reaction by SW may have some positive effect on angiogenesis during bone regeneration.

Angiogenesis is an essential component of skeletal development, and vascular endothelial growth factor (VEGF)[1] signaling plays an important role in this process (1). VEGF is secreted in four biologically active isoforms that arise from alternative splicing of the VEGF primary transcription. VEGF-A is the most abundant of the four isoforms and is commonly used in studies investigating the biological effects of VEGF (2, 3). Exogenous VEGF was found to enhance blood vessel formation, ossification, and new bone (callus) maturation in mouse femur fractures (4). Targeted deletion of VEGF gene caused skeletal defect in mice by impairing angiogenesis and endochondral bone formation (5). During bone formation, there is a cross-talk between endothelial cells and osteoblasts. VEGF was reported to induce endothelial cell migration, proliferation, and capillary permeability of vascularization process in endochondral bone (6). These findings indicate that osteoblast-derived VEGF in the bone microenvironment has an important role in angiogenic activities during bone formation.

Bone regeneration and blood vessel formation of fractured callus can be promoted by physical modalities (7, 8). Acoustic energy and pressure released by shock waves (SW) have been shown to have a positive effect on fracture healing and tendon repair (9–11), and mechanical stimulation has been found to raise adaptive modeling response of bone microenvironment via induction of anabolic molecules (12, 13). Previous studies have demonstrated that SW can stimulate bone marrow mesenchymal stem cell differentiation into osteoprogenitors, which has been associated with increases in osteogenic factor expression (15). Moreover, several cell types have been reported to respond to mechanical stimulation by elevating VEGF mediation of angiogenic responses (15, 16). In animal studies, we have also demonstrated that SW treatment can induce neovascularization of tendon-bone junction and may be associated with increases in VEGF-A expression (17). However, the exact

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; SW, shock wave; ERK, extracellular signal-regulated kinase; HIF-1α, hypoxia inducible factor-1α; CAM, chick chorioallantoic membrane; SOD, superoxide dismutase, L-NAME, *N*-nitro-L-arginine methyl ester; DPI, diphenyliodonium; RT-PCR, reverse transcription PCR; Ras, Rous sarcoma kinase; Raf-1, Rous sarcoma-associated factor-1; PEG, polyethylene glycol; ELISA, enzyme-linked immunosorbent assay; BMP, bone morphogenetic protein; TGF, transforming growth factor; IGF, ■.

molecular mechanism by which SW promotes angiogenesis has remained undetermined.

Hypoxia-inducible factor-1 (HIF-1), a heterodimeric basic helix-loop-helix transcription factor, acts as a critical regulator of VEGF expression. Active HIF-1 has been found to accumulate in the cell nucleus, bind to the target DNA sequence, and enhance hypoxia-inducible gene transcription (18). Evidence has suggested that reactive oxygen radicals mediate hypoxia, arsenite, and vanadate activation of HIF-1-dependent VEGF expression (19, 20). One of our recent studies has shown that superoxide mediated SW-promoted extracellular signal-regulated kinase (ERK) activation and mesenchymal stem cell differentiation into osteogenic lineage (21). This finding implies that induction of reactive oxygen molecules by SW potentially may provoke intracellular signaling transduction, which in turn may activate angiogenic activity of bone cells. We hypothesize that SW induce the reactive oxygen radical as a mediator in the activation of intracellular signal transduction and angiogenic transcription.

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The purposes of this study were to examine the effect of SW on the angiogenic response of human osteoblasts and to investigate whether SW promotion of angiogenic activity can be linked to the induction of reactive oxygen radicals, the activation of HIF-1α, and the promotion of VEGF-A production.

MATERIALS AND METHODS

Cell Culture—Human fetal preosteoblastic cells (CRL-11372, American Type Culture Center, Manassas, VA) were maintained in a mixture of phenol red-free Ham's F12 medium and Dulbecco's modified Eagle's medium (1:1) containing 10% fetal bovine serum and 2.5 mM L-glutamine (Invitrogen) in a 5% CO_2, 34 °C incubator for 6 days. Cells were harvested by trypsinization and resuspended in medium for further studies. Cell viability was determined using trypan blue exclusion.

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Shock Wave Treatment—Cells (1×10^6 cells/dish, 35-mm Petri dishes) were cultured for 48 h and subjected to SW treatment using OssaTron® SW equipment (HMT High Medical Technologies, Kreuzlingen, Switzerland) as described previously (14). Briefly, culture dishes were floated in a thermostastically controlled water bath. A sterile SW probe was immersed vertically into each culture dish and placed to just touch the surface of the medium. The distance between the probe and the cells was ~5–6 mm. Cells were exposed to a single SW treatment at 0.16 mJ/mm^2 energy flux density, 1 Hz, 500 impulse. This SW energy enhances osteogenic differentiation of mesenchymal stem cells (14). Control samples were prepared in the same manner except that they were not exposed to SW. Cells were harvested at 15 min, 30 min and 1, 3, 6, 12, and 24 h after SW treatment for RT-PCT and immunoblotting. To verify whether SW-augmented VEGF expression was regulated by osteogenic factors, SW-treated osteoblasts were co-cultured with or without 10 ng/ml monoclonal antibodies against BMP-2, TGF-β1, and IGF-I (R&D Systems) for 24 h, respectively. To investigate the role of bioactive radical in SW promotion of VEGF expression, subconfluent cell cultures were pretreated with or without 500 units/ml polyethylene glycol (PEG)-coupled bovine erythrocyte superoxide dismutase (SOD) and PEG-catalase (Sigma) to scavenge superoxide and hydrogen peroxide. To determine whether prostaglandin E_2 was involved in the SW-enhanced VEGF expression, cells were pretreated with or without 10 μM indomethacin to suppress PGE_2 production by inhibiting cyclooxygenase-2 activity. To elucidate the role of nitric oxide in the SW-promoted VEGF expression, osteoblasts were pretreated with or without 100 μM *N*-nitro-L-arginine methyl ester (L-NAME, Sigma) to inhibit nitric oxide production. To differentiate which oxidase was responsible for SW-induced superoxide production, osteoblasts were pretreated with 30 μM DPI (an NADPH oxidase inhibitor), 50 μM allopurinol (a xanthine oxidase inhibitor) or 50 μM rotenone (a mitochondrial oxidase inhibitor; Sigma). In some experiments, subconfluent cell cultures were pretreated with 20 μM PD98059 (Calbiochem) to inhibit ERK activity for 4 h before SW treatment.

RT-PCR—Total RNA was extracted and purified from 10^6 cells with and without SW treatment using Tri-reagent (Sigma). One microgram of total RNA was reverse transcribed into cDNA followed by PCR amplification using human gene-specific primers: VEGF-A, forward, 5′-TTA TAC CGG GAT TTC TTG CG-3′), and reverse, 5′-CCC ACT GAG GAG TCC AAC AT-3′ (209 base pair expected); β-actin, forward, 5′-CGC CAA CCG CGA GAA GAT-3′, β-actin reverse, 5′-CGT CAC CGG AGT CCA TCA-3′) (168 base pair expected). The parameters for RT-PCR cycling were set as described previously (14). All signals were quantified by scan densitometry, and the final value was obtained by calculating the VEGF-A/β-actin ration value. The -fold of promotion was calculated as the increase over the value of its corresponding control sample.

Determination of Superoxide Production—Superoxide production by cell cultures with or without SW was determined using a horse heart cytochrome *c* reduction assay in the absence and presence of SOD and calculated from the molar extinction coefficient of 0.0282 $\mu M^{-1}cm^{-1}$ as described previously (21).

Measurement of VEGF-A Production in the Culture Supernatants—The VEGF-A levels of culture supernatant were determined using ELISA kits (Quantikine®, R&D Systems) according to manufacturer's instructions. Results were calculated using an interpolation determined from a standard curve made by a series of VEGF-A concentrations.

Transient Transfection of a Dominant Negative Ras Plasmid—cDNAs encoding wild type and mutant (S17N) H-Ras proteins (DN-Ras) were ligated and cloned into pUSE vectors (Upstate Biotechnology, Lake Placid, NY), respectively. Stable transfection and selection were accomplished according to previously described methods (22). Briefly, osteoblasts (5×10^5 cells/well, 6-well plate) were plated to reach 60–80% confluence. Cells were transfected with 5 μg of wild type Ras and DN-Ras mutant cDNA plasmid (14) using FuGENE™ 6 transfection reagent (Roche Diagnostics) according to manufacturer's instructions. Cells stably transfected with the plasmid were selected in medium containing 600 μg/ml G418 (Invitrogen).

Cytosolic and Nuclear Extracts—Cytosolic and nuclear extracts of cell cultures were prepared as described previously (21). Briefly, cytosolic extracts were harvested by lysing cells with buffer containing 10 mM Tris-HCl (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 0.7% Nonidet P-40 for 10 min at 4 °C and centrifuged at $500 \times g$ for 5 min, and then supernatants were harvested. Pellets were further lysed with buffer containing 40 mM Tris-HCl (pH 7.9), 350 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM dithiothreitol, 2 μg/ml leupeptin, and 1 μg/ml aprotinin for 20 min at 4 °C, and supernatants were harvested after centrifugation at $12{,}000 \times g$ at 4 °C for 10 min. Protein concentrations in cytosolic and nuclear extracts were determined using a Bio-Rad assay kit.

Measurement of Ras and Rac1 Activation by Raf-1 and PAK-1 Agarose Conjugate—Ras and Rac1 activation were determined using Ras and Rac1 activation assay kits (Upstate Biotechnology). Briefly, cell lysates were precleared with glutathione-agarose followed by incubation with specific Raf-1 RBD and PAK-1 PBD agarose conjugates, respectively. The immunoprecipitates were reacted with Laemmli buffer containing 200 mM Tris, pH 6.8, 10% glycerol, 4% SDS, 50 μM dithiothreitol, and 0.05% bromphenol blue for 5 min at 95 °C. The mixtures were subject to Western blot assay. Activated Ras and Rac1 proteins on the blot were recognized by a mouse anti-Ras and anti-Rac1 antibodies (Upstate Biotechnology) followed by goat anti-mouse horseradish peroxidase-conjugated IgG as the second antibody. The activated Ras protein and Rac1 levels were visualized using chemiluminescence agents (SuperSignal®, Pierce) (14).

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Determination of ERK, p38, and HIF-1α Phosphorylation—Cytosolic and nuclear extracts (500 μg) were reacted with anti-ERK, anti-p38 antibodies (Upstate Biotechnology), and HIF-1α antibodies (Santa Cruz Biotechnology) and were precipitated with protein A (Sigma), respectively. Immunoprecipitates (20 μg) were subjected to Western blot assay (21). Total ERK, p38, and HIF-1α on the blots were recognized by anti-ERK, anti-p38, and HIF-1α antibodies followed by horseradish peroxidase-conjugated IgG as the second antibody and were visualized with chemiluminescence agents. The phosphorylated ERK, p38, and HIF-1α were recognized by stripping the membrane in a buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM mercaptoethanol for 30 min at 50 °C and then reprobed with mouse anti-phospho-ERK, anti-phospho-p38, and anti-phosphotyrosine antibodies (Upstate Biotechnology) , respectively, using a similar procedure.

Electrophoretic Mobility Shift Assay—A HIF-1 consensus VEGF-A promoter (underlined) oligonucleotides probe (5′-CCA CAG CAT ACG TGG GCT CCA ACA-3′, 3′-GGT GTC GTA TGC ACC CGA GGT TGT-5′) was 5′ end-labeled with γ-^{32}P using T4 polynucleotide kinase (New England Biolabs Inc.) and [γ-^{32}P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham Biosciences). Nuclear extracts (10 μg) were incubated with a binding buffer containing 10 mM HEPES (pH 7.9) 1 mM dithiothreitol, 1 mM EDTA, 80 mM KCl, 20% glycerol, and 0.25 mg/ml poly(dI-dC) (Amersham Biosciences) and 1.75 pmol of γ-^{32}P-labeled oligonucleotide probe (30000–40000 cpm; 2 μl). To specify protein/DNA binding reac-

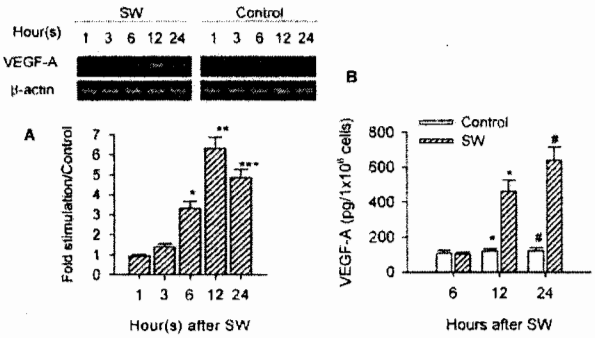

FIG. 1. **SW promoted VEGF-A mRNA expression of human osteoblasts in 6 h, peaking at 12 h.** Osteoblasts (1×10^6 cells/dish, 35-mm Petri dishes) were treated with SW at 0.16 mJ/mm^2, 1 Hz, 500 impulses. Cells were subjected to assessment of VEGF mRNA 1, 3, 6, 12, and 24 h after SW treatment. After standardization of housekeeping gene expression, equal amount of cDNA from each sample were subjected to 40 cycles of PCR to amplify VEGF-A mRNA expressions. * (p = 0.015), ** ($p < 0.001$), and *** ($p < 0.001$) indicate a significant difference between SW and control groups (*A*). SW enhanced VEGF-A protein production in 12 h. VEGF-A levels in culture supernatants of osteoblasts with and without SW treatment were determined by ELISA. * (p = 0.022) and # (p = 0.006) indicate a significant difference between two groups (*B*). Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

tions, 1 μl of anti-HIF-1α antibodies was added to binding buffer, incubated for 30 min at 4 °C, and mixed with γ-^{32}P-labeled oligonucleotide probe. Samples were electrophoresed through 6% polyacrylamide gel in 0.5% TBE (45 mM Tris, 45 mM boric acid, 10 mM EDTA, pH 8.3). The gel was dried and the radioactive band visualized using Kodak Bio-Max film with an intensifying screen at −70 °C.

In Vivo Angiogenesis Assay—Concentrated culture supernatants were harvested by lyophilizing 1 ml of culture supernatants of osteoblasts with and without SW treatment in the presence or absence of 500 units/ml PEG-SOD and then resuspending them in 20 μl of phosphate-buffered saline. VEGF-A concentrations in the mixtures were determined using a VEGF-A ELISA kit. To confirm whether VEGF-A was involved in angiogenesis, culture supernatants were neutralized with 50 ng/ml monoclonal anti-VEGF-A antibodies (R&D Systems). Concentrated culture supernatants were subjected to assessment of angiogenesis using a chick chorioallantoic membrane (CAM) assay as described previously (23). Briefly, fertile White Leghorn chicken eggs were incubated at 37 °C and 70% relative humidity. On the third day of incubation, the eggs were windowed by gentle sanding to expose an opening on the CAM. The openings were sealed with UV-sterilized adhesive tape, and the eggs were further incubated until day 5. On day 5, silastic rings were placed on the CAM surface. Concentrated culture supernatants were applied inside the rings. The eggs were resealed and incubated for 5 days. CAMs were examined and photographed with a Nikon color camera under microsurgery microscope. A vascular index was determined by counting all discernible vessels traveling the ring and was expressed as the relative increase of the number of vessels under different conditions in comparison with the control (23).

Statistical Analysis—All values were expressed as mean ± S.E. Student's paired t test was used to evaluate the difference between the sample of interest and its respective control. For analysis of the time course, a multiple range of analysis of variance was used. A p value of <0.05 was considered significant.

RESULTS

SW Treatment Raised VEGF-A Expression—We first determined whether SW augmented VEGF-A gene expression in osteoblasts. Cell cultures were treated with SW at 0.16 mJ/mm^2 energy flux density, 1 Hz, 500 impulses. There was no significant difference in cell viability between SW and control groups (data not shown). RT-PCR results indicated that VEGF-A mRNA expression significantly increased in 6 h, peaking at 12 h (Fig. 1*A*). ELISA results also showed that osteoblasts subjected to SW treatment significantly increased VEGF-A production in 12 h (Fig. 1*B*).

SW Promotion of VEGF-A Expression Mediated by Superoxide but Not by Osteogenic Factors or Nitric Oxide, Hydrogen Peroxide, or Prostaglandin E_2—Previous studies have demonstrated that osteogenic factors can regulate VEGF expression of osteoblasts (24–26) We sought to elucidate whether osteogenic factors were involved in SW-augmented VEGF expression, SW-treated osteoblasts were co-cultured with BMP-2, TGF-β1, and IGF-I monoclonal antibodies for 24 h, respectively. BMP-2, TGF-β1, IGF-I neutralization did not significantly alter SW-enhanced VEGF-A mRNA expression (Fig. 2*A*) or VEGF-A production (Fig. 2*B*). The accumulated evidence suggests that cells responded to SW by altering biological activities through influx of bioactive molecules such as oxygen radicals or PGE_2 (21, 27). We investigated which reactive radical might be responsible for mediating SW increase in VEGF-A expression. Scavenging of hydrogen peroxide by 500 units/ml PEG catalase did not influence SW-promoted VEGF-A expression. Inhibition of cyclooxygenase-2 activity by 10 μM indomethacin and nitric oxide synthase activity by 100 μM L-NAME did not affect SW-promoted VEGF-A mRNA expression (Fig. 2*C*) or VEGF-A production (Fig. 2*D*). Nevertheless, PEG-SOD pretreatment (500 units/ml) significantly reduced SW enhancement of VEGF-A expression (Fig. 2*C*) and VEGF production (Fig. 2*D*). These findings suggest that superoxide, but not nitric oxide, hydrogen peroxide, or prostaglandin E_2, was involved in the ESW promotion of VEGF-A expression.

DPI Pretreatment Reduced SW-induced Superoxide and VEGF-A Expression—Osteoblasts with SW treatment significantly increased superoxide production in 30 min. This higher production of superoxide persisted for 24 h (Fig. 3*A*). We determined whether SW promotion of superoxide production was linked to mitochondrial oxidase, xanthine oxidase, or NADPH oxidase. Pretreatment with DPI (an NADPH oxidase inhibitor), but not with other oxidase inhibitors, significantly reduced SW-promoted superoxide production (Fig. 3*B*), VEGF-A mRNA expression (Fig. 3*C*), and VEGF-A production (Fig 3*D*). This suggests that NADPH oxidase was responsible for SW-augmented superoxide production.

Ras Regulated SW-promoted Rac1 Activation, Superoxide Production, and VEGF-A Expression—There has been some evidence that Ras and Rac1 proteins are involved in NADPH oxidase-derived superoxide synthesis (28, 29). We sought to examine whether SW-induced superoxide production could be associated with Ras or Rac1 activation. SW rapidly activated Ras protein in 15 min and Rac1 protein in 30 min (Fig. 4*A*), respectively. To verify whether SW promotion of superoxide production and VEGF-A expression was regulated by Ras protein, we subjected wild type Ras- and dominant negative Ras-transfected osteoblasts to SW treatment. Transfection of the mutant-Ras completely reduced SW-induced Ras and Rac1 activation (Fig. 4*B*). Superoxide synthesis (Fig. 4*C*) and VEGF-A mRNA expression (Fig. 4*D*) were also significantly suppressed in the mutant Ras-transfected cells.

SW-promoted Phosphorylation of ERK and HIF-1α—Experiments were done to elucidate whether SW-increased VEGF-A expression was linked to mitogen-activated protein kinase and angiogenic transcription. Immunoblotting indicated that SW increased ERK activation in 1 h as demonstrated by phosphorylated ERK expression (Fig. 5*A*). SW did not affect p38 phosphorylation throughout the study period (Fig. 5*B*). Furthermore, SW increased nuclear HIF-1α phosphorylation, as demonstrated by phosphotyrosine expression of HIF-1α, in 3 h (Fig. 6*A*) and promoted HIF-1 binding to VEGF-A promoter, as determined by electrophoretic gel shift (Fig. 6*B*). We employed monoclonal antibodies against HIF-1α to confirm the DNA-protein binding activity. An electrophoretic mobility shift assay

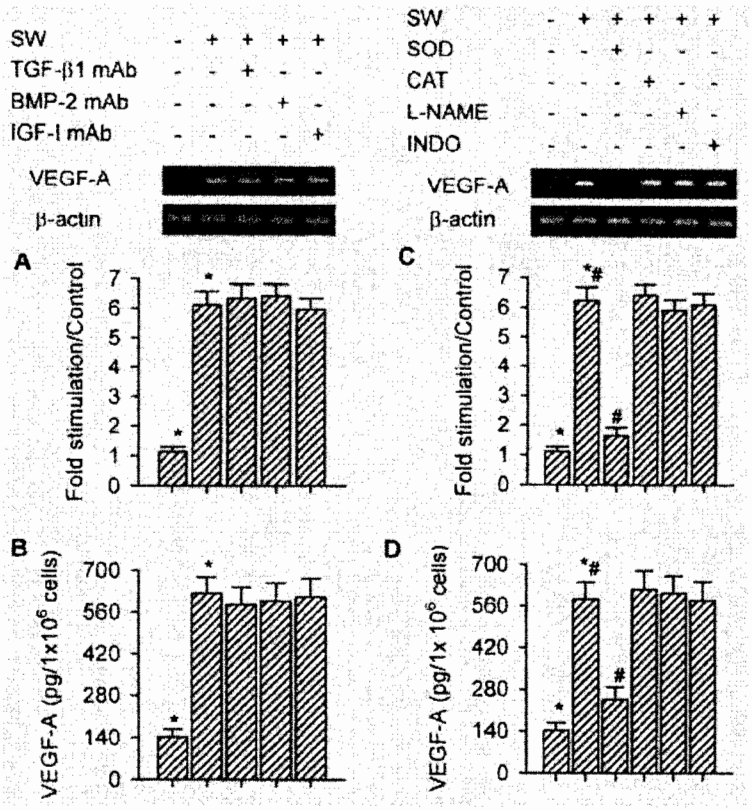

FIG. 2. *A* and *B*, SW-promoted VEGF-A mRNA expression (*A*) and VEGF-A production (*B*) were not affected by BMP-2 or TGF-β1 or IGF-I. Osteoblasts were co-cultured with or without 10 ng/ml BMP-2, TGF-β1 or IGF-I monoclonal antibody for 12 h after SW treatment. * ($p < 0.001$) indicates a significant difference between two groups. *C* and *D*, superoxide, but not hydrogen peroxide, prostaglandin E_2, or nitric oxide, was involved in ESW promotion of VEGF-A mRNA expression (*C*) and VEGF-A production (*D*). Cells (1×10^6 cells/dish, 35-mm Petri dishes) were treated with 500 units/ml PEG-SOD (500 units/ml), PEG-catalase (500 units/ml), indomethacin (*INDO*, 10 μM), or 100 μM L-NAME for 1 h and exposed to ESW treatment at 0.16 mJ/mm^2 for 500 impulses. Cells were subjected to assessment of VEGF mRNA 12 h after treatment. After standardization of housekeeping gene expression, equal amounts of cDNA form each sample were subjected to 40 cycles of PCR to amplify VEGF-A expression. VEGF-A levels in culture supernatants of osteoblasts 24 h after SW treatment were assessed by ELISA. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

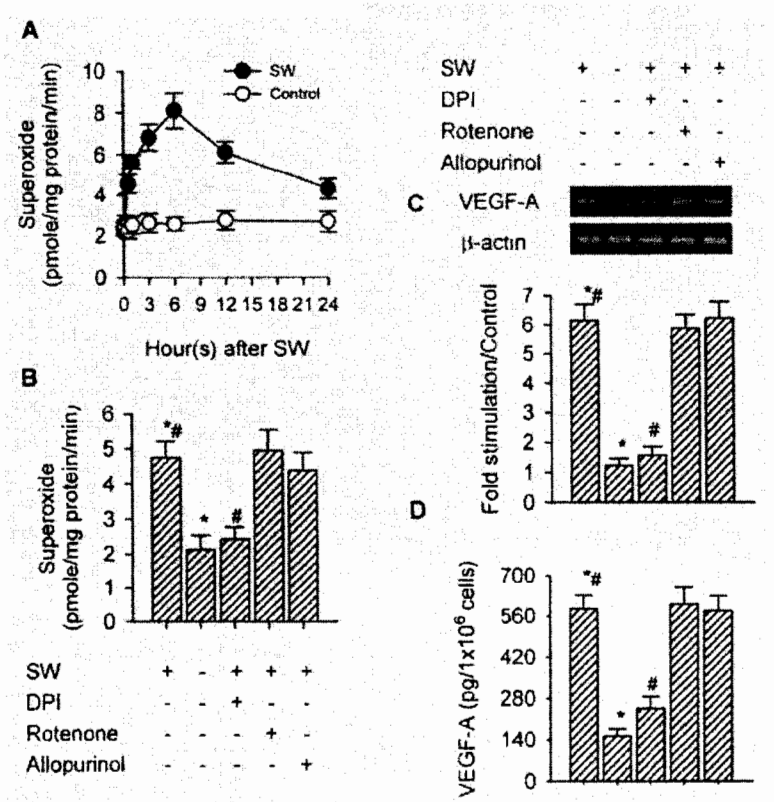

FIG. 3. SW significantly increased superoxide production 30 min, 1, 3, 6, 12, and 24 h after treatment (*A*). Inhibition of superoxide by DPI, but not by rotenone or allopurinol, reduced SW-augmented superoxide production. Cells (1×10^6 cells/dish, 35-mm Petri dishes) were treated with 30 μM DPI, 50 μM rotenone, or 50 μM allopurinol and exposed to SW treatment at 0.16 mJ/mm^2 for 500 impulses. Cells were subjected to assessment of superoxide production 30 min following SW. * ($p < 0.001$) and # ($p < 0.001$) represent a significant difference between two groups (*B*). DPI pretreatment reduced SW-augmented VEGF-A mRNA expression in 12 h. After standardization of housekeeping gene expression, equal amount of cDNA form each sample were subjected to 40 cycles of PCR to amplify VEGF-A expression. * ($p = 0.008$) and # ($p < 0.001$) indicate a significant difference between two groups (*C*). Inhibition of superoxide by DPI, but not by rotenone or allpurinol, reduced SW-augmented VEGF-A production * ($p < 0.001$) and # ($p = 0.019$) indicate a significant difference between two groups (*D*). Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

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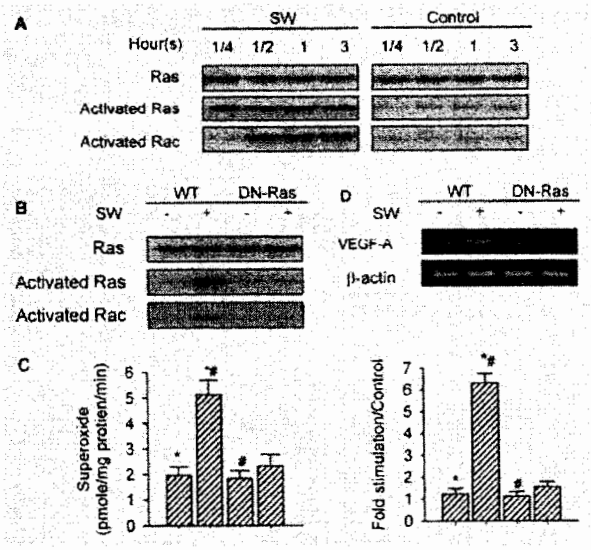

FIG. 4. **Ras and Rac activation in SW-treated osteoblasts.** SW activated Ras protein in 15 min and Rac protein in 30 min. Cell cultures with and without SW treatment were subjected to immunoprecipitation and immunoblotting (*A*). Transfection of dominant negative Ras mutant suppressed SW-enhanced Ras and Rac activation in 15 and 30 min, respectively (*B*). Dominant negative Ras mutant reduced SW-induced superoxide production in 30 min. * ($p < 0.001$) and # ($p < 0.001$) represent a significant difference between two groups (*C*). Dominant negative Ras mutant abrogated SW-enhanced VEGF-A mRNA expression in 12 h. After standardization of housekeeping gene expression, equal amounts of cDNA from each sample were subjected to 40 cycles of PCR to amplify VEGF-A expression. * ($p < 0.001$) and # ($p < 0.001$) indicate a significant difference between two groups (*D*). Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

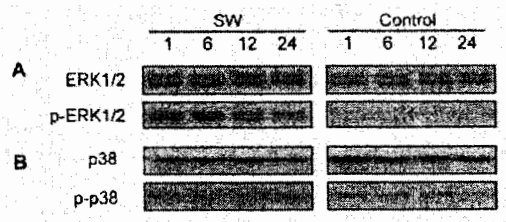

FIG. 5. **SW activated ERK but not p38 phosphorylation.** SW raised ERK phosphorylation in 1 h (*A*). SW did not alter p38 activation (*B*). ERK and p38 immunoprecipitates harvested from cytosolic extract of osteoblasts with and without SW treatment were subjected to immunoblotting. Phosphorylated ERK and p38 were probed with anti-phosphorylated ERK and phosphorylated p38 antibodies, respectively.

radiograph showed that nuclear extract harvested from SW-treated osteoblasts was indeed super-shifted by anti-HIF-1α antibodies (Fig. 6*C*). These findings indicate that SW activates HIF-1α binding to the VEGF promoter.

SOD and PD98059 Pretreatments Reduced SW-augmented ERK and HIF-1α Activation—Scavenging of superoxide by PEG-SOD (500 units/ml) and inhibition of ERK activation by PD98059 significantly decreased SW-activated ERK and HIF-1α phosphorylation (Fig. 7*A*). Furthermore, scavenging of superoxide and inhibition of ERK activity abrogated SW-enhanced HIF-1α binding to VEGF-A promoter (Fig. 7*B*), VEGF-A mRNA expression (Fig. 7*C*), and VEGF-A production (Fig. 7*D*). These results suggest that superoxide plays an important role in the regulation of SW-augmented ERK, HIF-1α activation, and VEGF-A expression of osteoblasts.

SW Stimulated Angiogenesis through Superoxide-mediated VEGF-A—Using a CAM assay, we tested whether SW could induce *in vivo* angiogenesis through superoxide-mediated VEGF-A. Culture supernatant from SW-treated osteoblasts significantly increased angiogenesis of CAM, which was visible through a microscope as a brush-like pattern of blood vessels (Fig. 8*A*) in comparison with the control without SW treatment (Fig. 8*B*). VEGF-A monoclonal antibody neutralization (Fig. 8*C*) and SOD scavenging of superoxide significantly reduced SW-induced angiogenic response (Fig. 8*D*).

DISCUSSION

In this study, elevation of VEGF mRNA and protein levels in osteoblasts was found to follow physical SW treatment, which rapidly induced Ras-dependent superoxide production. This reactive oxygen biomolecule plays a critical role in regulating cytosolic ERK phosphorylation and HIF-1α transactivation. Mechanisms underlying SW-augmented angiogenesis in the musculoskeletal system are not well understood. Our findings provide the first indication that osteoblasts respond to SW by raising intracellular angiogenic signal transduction and angiogenesis through superoxide-mediated VEGF-A induction, providing a clear molecular explanation for SW-promoted angiogenesis and neovascularization of bone tissue. These findings agree with those in our previous study, which demonstrated that SW raises the anabolic responses of osteogenic cells (14). We propose that it is the increased angiogenic response of osteoblasts that brings about the clinical success of SW treatment in the promotion of fracture healing.

One of our previous animal studies demonstrated that segmental femoral defects that receive SW treatment undergo progressive bone formation and vascularization in healing process, indicating that angiogenic activities potentially are increased in bone tissue subjected to SW stimulation (30). In this study, we have further shown that SW elicit a time-dependent effect on VEGF-A expression. Increasing the blood vessel formation of CAM provided *in vivo* evidence that osteoblasts can convert biophysical SW stimuli into angiogenic responses by increasing VEGF-A production. VEGF is an important molecule for endothelial cell proliferation and elicits proliferative and chemotactic effects on osteoblasts (31). In contrast to other studies implying that VEGF expression of osteoblasts was regulated by osteogenic factors (24–26), we have shown that IGF-I, TGF-β1, or BMP-2 neutralization does not alter SW promotion of VEGF-A expression. We speculate that the difference lies in the possibility that these osteogenic factors probably had not yet participated in regulating VEGF expression of SW-treated osteoblasts in the study period (24 h). Up-regulation of angiogenic activity probably came about as a result of SW-sensitive bioactive mediator or intracellular signal transduction.

Physical SW have been found to disturb cell homeostasis and bioactive molecule influx (32), possibly indicating that bioactive molecules may initialize intracellular signal. A growing body of evidence has suggested that reactive radicals are involved in regulating VEGF expression (33, 34). In our current study, we found that superoxide, but not hydrogen peroxide, prostaglandin E_2, or nitric oxide, acted as a potent mediator for VEGF-A expression of osteoblasts following SW treatment. Enhancement of superoxide production can be derived from the oxidation chain reactions in mitochondria, xanthine oxidase activity in cytosol, or NADPH oxidase in the plasma membrane. We suggest that NADPH oxidase was responsible for SW-elevated superoxide production, because superoxide synthesis and VEGF-A expression was inhibited by DPI but not by rotenone or allopurinol. In contrast to other studies demonstrating that superoxide-induced oxidative stress causes cell injury (35), we noted that SW-induced superoxide activated the anabolic responses of osteogenic cells by increasing osteogenic and angiogenic factors. Previous studies have indicated that low level generation of reactive oxygen rad-

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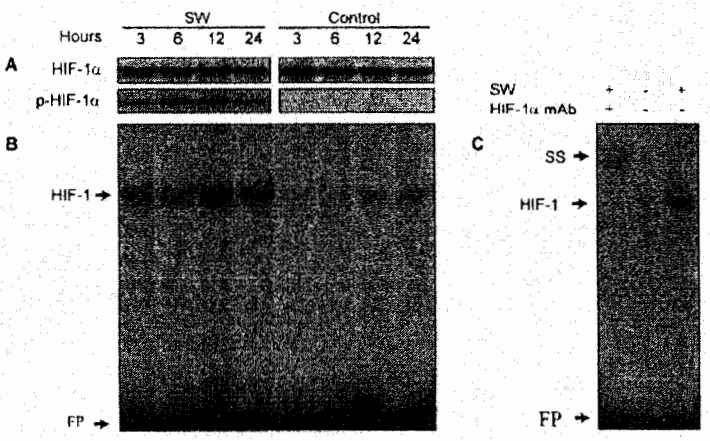

FIG. 6. **SW-induced HIF-1α activation.** *A*, SW activated nuclear HIF-1α phosphorylation in 3 h. Nuclear HIF-1α immunoprecipitates isolated from nuclear extracts of osteoblasts with and without SW treatment were subjected to immunoblotting. Phosphorylated HIF-1α was probed using anti-phosphotyrosine antibodies. SW promoted binding activity of HIF-1α with VEGF-A promoter in 3 h as determined by electrophoretic mobility shift assay. *B*, supershift of HIF-1α. Nuclear extracts of SW-treated osteoblasts were incubated with HIF-1 probe in the presence or absence of anti-HIF-1α antibodies. *FP*, free probe, *SS*, supershift.

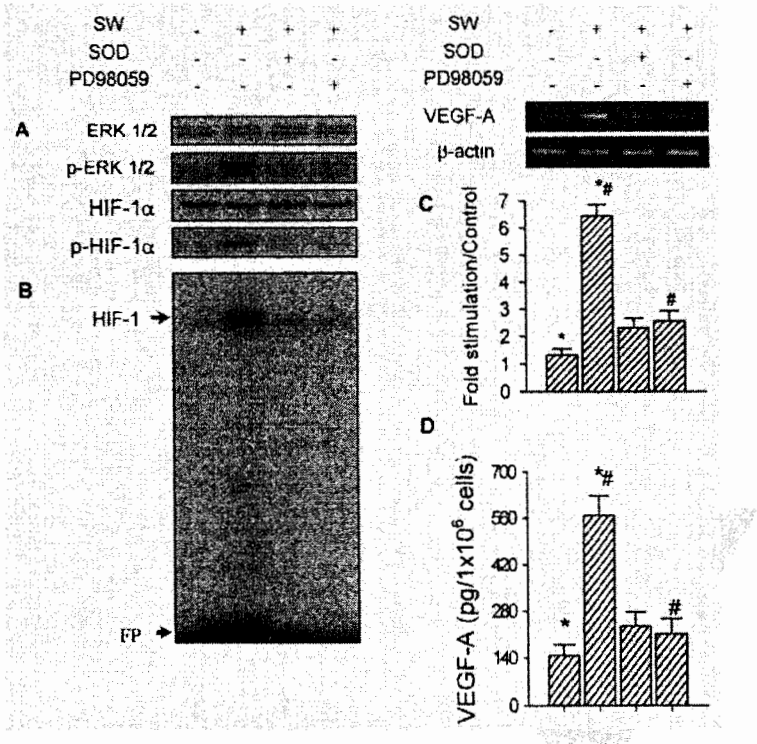

FIG. 7. Scavenging of superoxide by SOD and inhibition of ERK activity by PD98059 suppressed SW-activated cytosolic ERK activation in 1 h and HIF-1α phosphorylation in 3 h (*A*) and binding activity of HIF-1α with VEGF-A promoter in 3 h (*B*). *C*, scavenging of superoxide by SOD and inhibition of ERK activity by PD98059 reduced SW-promoted VEGF mRNA expression in 12 h. * ($p < 0.001$) and # ($p = 0.003$) indicate a significant difference between two groups. *D*, scavenging of superoxide by SOD and inhibition of ERK activity by PD98059 reduced SW-promoted VEGF production in 24 h. * ($p < 0.001$) and # ($p = 0.012$) indicate a significant difference between two groups. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

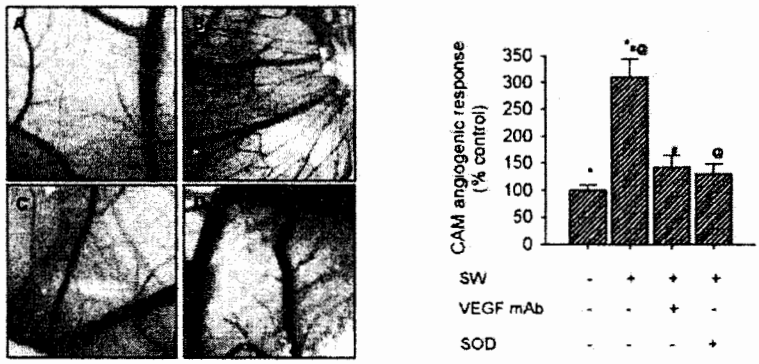

FIG. 8. **Culture supernatant of SW-treated osteoblasts is angiogenic in the CAM assay.** SW enhanced blood vessel formation of CAM (*A*) in comparison with the control without SW treatment (*B*). *C*, neutralization of VEGF-A with monoclonal VEGF-A antibody reduced SW-promoted angiogenesis. *D*, scavenging of superoxide by SOD decreased SW-augmented angiogenic responses. *Right panel*, * ($p = 0.012$), # ($p = 0.021$), and @ ($p = 0.018$) indicate a significant difference between two groups. Results were obtained from 12 eggs. CAMs were photographed 5 days after incubation with culture supernatants.

icals in nonphagocytic cells participates in a diverse number of normal physiological or cell signals (36). Our findings support the general concept that a family of NAD(P)H-dependent oxidoreductases may be present in nonphagocytic cells that function as generators of redox signal in response to various growth factor and physical stimulation.

Previous research has also demonstrated that SW induce cell membrane hyperpolarization, implying that osteoblasts respond to physical SW stimulation by initialization of membrane-bound signal transduction (14). In the current study, SW rapidly activated Ras protein and subsequently induced Rac1 activation and elevated superoxide production. We suggest that the Ras and Rac1 pathway is, at least in part, responsible for superoxide production after SW stimulation. NADPH oxidase is a multicomponent enzyme complex located in the plasma membrane and is triggered by Ras and Rac1 proteins. Rac1 protein has also been found to be involved in the physical stimulation of superoxide synthesis (37–39). Moreover, Ras activation is required for VEGF expression, and inhibition of Ras signal transduction suppresses VEGF induction of angiogenic phenotype (40). Ras protein seems to act as a mechanosensitive molecule in the adaptation of the osteoblasts to SW-induced superoxide and VEGF expression, because SW-augmented Rac1 activation, superoxide production, and VEGF expression were completely reduced in dominant negative Ras-transfected cells.

We have further demonstrated in this study that SW-induced superoxide might activate cytosolic ERK, subsequently activating nuclear HIF-1α phosphorylation and promoting HIF-1α binding to VEGF-A promoter. The mitogen-activated protein kinase (MAPK) family has been reported to play an important role in oxidant-mediated HIF-1α-dependent VEGF expression (41). Previous studies have shown ERK to be a target molecule for mechanical stimulation of osteoblast proliferation (42, 43). SW treatment induced time-dependent ERK phosphorylation. PD98059 inhibition of ERK activity and scavenging of superoxide by SOD has been shown to reduce SW-enhanced VEGF-A expression. We suggest that ERK is an important pulsed acoustic energy- and oxidant-sensitive regulator able to transmitting biophysical signal imparted by SW into the nucleus to activate a cascade of angiogenic transcription. Moreover, SW-activated HIF-1α and angiogenic responses of osteoblasts come about through superoxide- and ERK-dependent pathways. Our findings support previous studies showing that reactive oxygen species are involved in HIF-1α stabilization, nuclear translocation, and activation (44). Although these data demonstrate that VEGF-A production is essential for SW-enhanced angiogenesis, we cannot exclude the possibility that other angiogenic factors, such as fibroblasts growth factor and platelet-derive growth factor, may contribute to this effect. Our research findings emphasize that VEGF-A expression in osteoblasts can be regulated directly by physical stimuli and redox reactions.

Bone cells convert biophysical stimuli into biochemical responses that alter gene expression and cellular adaptation. SW rapidly activated membrane bound Ras and Rac1 proteins of osteoblasts, indicating that bioactive molecules located at the cell surface, such as G proteins and extracellular integrin, may be activated by acoustic energy released by SW. Further studies are needed to explore the role of these molecules in regulating SW-induced angiogenic response of osteoblasts. Taken together, we have provided evidence that regulation of redox reactions by biophysical factors such as SW might provide a promising regimen for regulating ERK signal transduction and activating the angiogenic transcription factor, HIF-1α, resulting in an increase in VEGF-A production and blood vessel formation in CAM. By using the SW-induced signal transduction pathway for angiogenesis, the biopharmacological modulation of fracture healing and wound healing may also be possible.

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REFERENCES

1. Deckers, M. M. L.M. M. L., Karperien, M., van der Bent, C., Yamshita, T., Papapoulos, S. E., and Lowik C. W. G. M. (2000) *Endocrinology* **141,** 1667–1674
2. Ferrara, N. K., Houck, L., Jakeman, L., and Leung, D. W. (1992) *Endocr. Rev.* **13,** 18–32
3. Thomas, K. A. (1996) *J. Biol. Chem.* **271,** 603–606
4. Street, J., Bao, M., deGuzman, L., Bunting, S., Peale, F. V., Jr., Ferrara, N., Steinmetz, H., Hoeffel, J., Cleland, J. L., Daugherty, A., van Bruggen, N., Redmond, H. P., Carano, R. A., and Filvaroff, E. H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 9656–9661
5. Maes, C., Carmeliet, P., Moermans, K., Stockmans, I., Smets, N., Collen, D., Bouillon, R., and Carmeliet, G. (2002) *Mech. Dev.* **111,** 61–73
6. Ferguson, C., Alpern, E., Miclau, T., and Helms, J. A. (1999) *Mech. Dev.* **87,** 57–66
7. Aroson, J., Shen, X. C., Skinner, R. A., Hogue, W. R., Badger, T. M., and Lumpkin, C. K. (1997) *J. Orthop. Res.* **15,** 221–226
8. Azuma, Y., Ito, M., Harada, Y., Takagi, H., Ohta, T., and Jingushi, S. (2001) *J. Bone Miner. Res.* **16,** 671–680
9. Romp, J. D., Rosendahl, T., Schollner, C., and Theis, C. (2001) *Clin. Orthop.* **387,** 102–111
10. Wang, C. J., Chen, H. S., Chen, C. E., and Yang, K. D. (2001) *Clin. Orthop.* **387,** 95–101
11. Loew, M., Daecke, W., Kusnierczak, D., Rahmanzadeh, M., and Ewerbeck, V. (1999) *J. Bone Jt. Surg. Br.* **81,** 863–867
12. Burger, E. H., and Klein-Nulend, J. (1999) *FASEB J.* **13,** S102–S112
13. Salter, D. M., Wallace, W. H. B., Robb, J. E., Caldwell, H., and Wright, M. O (2001) *J. Bone Miner. Res.* **15,** 1746–1755
14. Wang, F. S., Wang, C. J., Huang, H. C., Chung, H., Chen, R. F., and Yang, K. D. (2001) *Biochem. Biophys. Res. Commun.* **287,** 648–655
15. Rivilis, I., Milkiewicz, M., Boyd, P., Goldstein, J., Brown, M. D., Egginton, S., Hansen, F. M., Hudlicka, O., and Haas, T. L. (2002) *Am. J. Physiol.* **283,** H1430–H1438
16. Shay-Salit, A., Shushy, M., Wolfovitz, E., Yahav, H., Breviario, F., Dejana, E., and Resnick, N. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 9462–9467
17. Wang C. J., Wang, F. S., Yang, K. D., Huang, C. S., Hsu, C. C., and Yang, L. C. (2003) *J. Orthop. Res.* **21,** 984–989
18. Guillemin, K., and Krasnow, M. A. (1997) *Cell* **89,** 9–12
19. Duyndam, M. C., Hulscher, T. M., Fontijn, D., Pinedo, H. M., and Boven, E. (2001) *J. Biol. Chem.* **276,** 48066–48076
20. Gao, N., Ding, M., Zheng, J. Z., Zhang, Z., Leonard, S. S., Liu, K. J., Shi, X., and Jiang, B. H. (2002) *J. Biol. Chem.* **277,** 31963–31971
21. Wang, F. S., Wang, C. J., Sheen-Chen, S. M., Kuo, Y. R., Chen, R. F., and Yang, K. D. (2002) *J. Biol. Chem.* **277,** 10931–10937
22. Feig, L. A., and Cooper, G. M. (1988) *Mol. Cell. Biol.* **8,** 3235–3243
23. Bellahcene, A., Bonjean, K., Fohr, B., Fedarko, N. S., Robey, F. A., Young, M. F., Fisher, L. W., Castronovo, V. (2000) *Circ. Res.* **86,** 885–891
24. Goad, D. L., Rubin, J., Wang, H., Tashjian, A. H., Jr., and Patterson, C. (1996) *Endocrinology* **137,** 2262–2268
25. Deckers, M. M. L., van Bezooijen, R. L., van der Horst, G., Hoogendam, J., van der Bent, C., Papapoulos, S. E., and Lowik, C. W. G. M. (2002) *Endocrinology* **143,** 1545–1553
26. Chua, C. C., Hamdy, R. C., and Chua, B. H. (2000) *Biochim. Biophys. Acta* **1497,** 69–76
27. Maier, M., Averbeck, B., Milz, S., Refior, H. J., and Schmitz, C. (2003) *Clin. Orthop.* **406,** 237–245
28. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Sulciner, D. J., Gutkind, J. S., Irani, K., Goldschmidt-Clermont, P. J., and Finkel, T. (1996) *Biochem. J.* **318,** 379–382
29. Irani, K., Xia, Y., Zweier, J. L., Sollott, S. J., Der, C. J., Feron, E. R., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont, P. J. (1997) *Science* **275,** 1649–1652
30. Wang, F. S., Kuo, Y. R., Yang, K. D., Huang, H. C., Wang, C. J., Sheen-Chen, S. M., and Chen, Y. R. (2003) *Bone* **32,** 387–396
31. Mayr-Wohlfart, U., Waltenberger, J., Hausser, H., Kessler, S., Gunther, K. P., Dehio, C., Puhl, W., and Brenner, R. E. (2000) *Bone* **30,** 472–477
32. Suhr, D., Brummer, F., Irmer, U., and Hulser, D. F. (1996) *Ultrasound Med. Biol.* **22,** 671–679
33. Liu, X. H., Kirschenbaum, A., Lu, M., Yao, S., Dosoretz, A., Holland, J. F., and Levine, A. C. (2002) *J. Biol. Chem.* **277,** 50081–50086
34. Kuroki, M., Voest, E. E., Amano, S., Beerepoot, L. V., Takashima, S., Tolentino, M., Kim, R. Y., Rohan, R. M., Colby, K. A., Yeo, K. T., and Adamis, A. P. (1996) *J. Clin. Investig.* **98,** 1667–1675
35. Durot, I., Maupoil, V., Ponsar, B., Cordelet, C., Vergely-Vandriesse, C., Rochette, L., and Athias, P. (2000) *Free Radic. Biol. Med.* **29,** 846–857
36. Funkel, T. (1998) *Curr. Opin. Cell Biol.* **10,** 248–253
37. Thannickal, V. J., Day, R. M., Klinz, S. G., Bastien, M. C., Larios, J. M., and Fanburg, B. L. (2000) *FASEB J.* **14,** 1741–1748
38. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G., and Segal, A. W. (1991) *Nature.* **353,** 668–670
39. Yeh, L. H., Park, Y. J., Hansalia, R. J., Ahmed, I. S., Deshpande, S. S., Goldschmidt-Clermont, P. J., Irani, K., and Alevriadou, B. R. (1999) *Am. J. Physiol.* **276,** C838—C847
40. Meadows, K. N., Bryant, P., and Pumiglia, K. (2001) *J. Biol. Chem.* **276,** 49289–49298
41. Mottet, D., Michel, G., Renard, P., Ninane, N., Raes, M., and Michiels, C. (1994) *J. Cell. Physiol.* **194,** 30–44
42. Wadhwa, S., Godwin, S. L., Peterson, D. R., Epstein, M. A., Raisz, L. G., and Pilbeam, C. C. (2002) *J. Bone Miner. Res.* **17,** 266–274
43. Hatton, J. P., Pooran, M., Li, C. F., Luzzio, C., and Hughes-Fulford, M. (2003) *J. Bone Miner. Res.* **18,** 58–66
44. Page, E. L., Robitaille, G. A., Pouyssegur, J., and Richard, D. E. (2002) *J. Biol. Chem.* **277,** 48403–48409