Ras Induction of Superoxide Activates ERK-dependent Angiogenic Transcription Factor HIF-1α and VEGF-A Expression in Shock Wave-stimulated Osteoblasts*

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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 media-

Angiogenesis is an essential component of skeletal development, and vascular endothelial growth factor (VEGF) 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) matura-

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/mm², 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 E₂, 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. Thus, modulation of redox reaction by SW may have some positive effect on angiogenesis during bone regeneration.

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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; T-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, insulin-like growth factor.
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. Oxygen, and angiogenic lineage induction (21). This finding elucidates the 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.

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 5% CO₂, 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. To determine whether prostaglandin E2 was involved in the SW- and PEG-catalase (Sigma) to scavenge superoxide and hydrogen peroxide. 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 secondary antibody and were visualized using chemiluminescence agents (SuperSignal®, Pierce) (14).

Determination of ERK, p38, and HIF-1α Phosphorylation—Cytosolic and nuclear extracts (500 μg) were reacted with anti-ERK, anti-p38 antibodies was added to binding buffer, 50 μl allogalpin (a xanthine oxidase inhibitor) or 50 μl p38 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 105 cells with and without SW treatment using Tri-reagent (Sigma). One microgram of total RNA was reverse transcribed by M-MLV reverse transcriptase (Promega) followed by PCR amplification using human gene-specific primers: VEGF-A, forward, 5′-TGA CCC GAA TCT TTC TGG CAG-3′, and reverse, 5′-GCC ACC CGA GGA GAT-3′; β-actin, forward, 5′-GCC ACC CGA GGA GAT-3′; β-actin reverse, 5′-GCC ACC CGA GGA GAT-3′ (168 base pair expected). The parameters for RT-PCR were as follows: denaturation at 95 °C for 30 min at 4 °C and centrifuged at 500 × g for 5 min, and then supernatants were harvested. Pellets were further lysed with buffer containing 40 μl Tris-HCl (pH 7.9), 350 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM dithiothreitol, 2 μM leupeptin, and 1 μg/ml aprotinin for 20 min at 4 °C, and supernatants were harvested after centrifugation at 12,000 × g at 4 °C for 10 min. Protein concentrations in cytosolic and nuclear extracts were determined by Bio-Rad assay kit.

Measurement of Ras and Rac1 Activation by Raf-1 and Pak1-Activated GTPase—Ras and Rac1 activation were determined using Ras and Rac1 activation assay kits (Upstate Biotechnology). Briefly, cells were extracted with ice-cold lysis buffer and nuclear extracts were harvested by lysing cells with buffer containing 50 μM Tris-HCl (pH 7.9), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, and 0.1% Nonidet P-40 for 10 min at 4 °C and centrifuged at 500 × g for 5 min, and then supernatants were harvested. Pellets were further lysed with buffer containing 40 μl Tris-HCl (pH 7.9), 350 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM dithiothreitol, 2 μM leupeptin, and 1 μg/ml aprotinin for 20 min at 4 °C, and supernatants were harvested after centrifugation at 12,000 × g at 4 °C for 10 min. Protein concentrations in cytosolic and nuclear extracts were determined by Bio-Rad assay kit.
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 resoled 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 ml/mm² 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. 1A). ELISA results also showed that osteoblasts subjected to SW treatment significantly increased VEGF-A production in 12 h (Fig. 1B).

SW Promotion of VEGF-A Expression Mediated by Superoxide but Not by Osteogenic Factors or Nitric Oxide, Hydrogen Peroxide, or Prostaglandin E₂—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, and IGF-I neutralization did not significantly alter SW-enhanced VEGF-A mRNA expression (Fig. 2A) or VEGF-A production (Fig. 2B). The accumulated evidence suggests that cells responded to SW by altering biological activities through influx of bioactive molecules such as oxygen radicals and PGE₂ (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 indo- methacin and nitric oxide synthase activity by 100 μM L-NAME did not affect SW-promoted VEGF-A mRNA expression (Fig. 2C) or VEGF-A production (Fig. 2D). Nevertheless, PEG-SOD pretreatment (500 units/ml) significantly reduced SW enhancement of VEGF-A mRNA and VEGF production (Fig. 2D). These findings suggest that superoxide, but not nitric oxide, hydrogen peroxide, or prostaglandin E₂, was involved in the SW 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. 3A). 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. 3B), VEGF-A mRNA expression (Fig. 3C), and VEGF-A production (Fig. 3D). 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. 4A), 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. 4B). Superoxide synthesis (Fig. 4C) and VEGF-A mRNA expression (Fig. 4D) 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 phospho-rylated ERK expression (Fig. 5A). SW did not affect p38 phosphorylation throughout the study period (Fig. 5B). Furthermore, SW increased nuclear HIF-1α phosphorylation, as demonstrated by phosphotyrosine expression of HIF-1α, in 3 h (Fig. 6A) and promoted HIF-1 binding to VEGF-A promoter, as determined by electrophoretic gel shift (Fig. 6B). We employed monoclonal antibodies against HIF-1α to confirm the DNA-protein binding activity. An electrophoretic mobility shift assay radiograph showed that nuclear extract harvested from SW-treated osteoblasts was indeed super-shifted by anti-HIF-1α antibodies (Fig. 6C). These findings indicate that SW activates HIF-1α binding to the VEGF promoter.
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 SW 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 SW 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 from 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 ml/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 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 (C). Inhibition of superoxide by DPI, but not by rotenone or allopurinol, 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.
Superoxide Induces Angiogenesis of Osteoblasts

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, 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.

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-augmented ERK and HIF-1α phosphorylation (Fig. 7A). Furthermore, scavenging of superoxide and inhibition of ERK activity abrogated SW-enhanced HIF-1α binding to VEGF-A promoter (Fig. 7B), VEGF-A mRNA expression (Fig. 7C), and VEGF-A production (Fig. 7D). 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. 8A) in comparison with the control without SW treatment (Fig. 8B). VEGF-A monoclonal antibody neutralization (Fig. 8C) and SOD scavenging of superoxide significantly reduced SW-induced angiogenic response (Fig. 8D).

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, prosstaglandin E2, 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 radicals 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 oxidoreduc-
tases 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

**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. B, SW promoted binding activity of HIF-1α with VEGF-A promoter in 3 h as determined by electrophoretic mobility shift assay. C, 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.
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 an important 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-derived 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 convey 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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