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Nitric oxide mediates ultrasound-induced hypoxia-inducible factor-1α activation and vascular endothelial growth factor-A expression in human osteoblasts

Feng-Sheng Wang,[a,*] Yur-Ren Kuo,[b] Ching-Jen Wang,[c] Kuender D. Yang,[a] Per-Rong Chang,[d] Yu-Ting Huang,[a] Hue-Chen Huang,[a] Yi-Chih Sun,[a] Ya-Ju Yang,[a] and Yeung-Jen Chen[e]

[a] *Department of Medical Research, Chang Gung Memorial Hospital, Kaohsiung, Taiwan*
[b] *Department of Plastic and Reconstructive Surgery, Chang Gung Memorial Hospital, Kaohsiung, Taiwan*
[c] *Department of Orthopedic Surgery, Chang Gung Memorial Hospital, Kaohsiung, Taiwan*
[d] *Fooyin University, Ta-Liao, Kaohsiung, Taiwan*
[e] *Department of Orthopedic Surgery, Chang Gung University, Linkou, Taiwan*

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Abstract

Vascular endothelial growth factor (VEGF) is an important regulator for angiogenesis and endochondral bone formation. Although low-intensity pulsed ultrasound (US) has been recently used for accelerating fracture healing, the effect of US stimulation on angiogenic factor production by osteoblasts remains undetermined. Here, we found that US elevation of VEGF-A expression in human osteoblasts to be mediated by nitric oxide (NO) and hypoxia-inducible factor-1α (HIF-1α). Human osteoblasts were treated with or without US stimulation (200 μs pulse, 1 kHz at 30 mW/cm^2) for 20 min. Cells were subjected to assessment of VEGF-A expression, NO production, nitric oxide synthase (NOS) catalytic activities, and HIF-1α transactivation. Results showed that US significantly increased VEGF-A mRNA and protein levels in 6 h. US augmentation of VEGF level was transcriptionally mediated. Early inhibition of NO production, but not calcium or prostaglandin E_2, significantly reduced US-enhanced VEGF-A levels. Osteoblasts responded to US treatment by increasing NO production, NOS catalytic activities, iNOS immunoexpression, nuclear HIF-1α activation, and binding to the VEGF-A promoter. Inhibition of NOS activity by *N*-nitro-L-arginine methyl ester (L-NAME) or blockade of guanylate cyclase activity by ODQ reduced US-augmented HIF-1α transactivation and VEGF-A levels. Conditioned medium harvested from US-treated osteoblasts promoted tube formation of human umbilical vein endothelial cells (HUVEC). Monoclonal VEGF-A antibody neutralization or L-NAME pretreatment reduced the promoting effect of conditioned medium on angiogenesis of HUVEC. Together, these findings show that NO plays an important role in mediating extracellular stimuli released by US and triggering intracellular response of osteoblasts to produce angiogenic factor after US treatment.

Keywords: Osteoblasts; Vascular endothelial growth factor; Hypoxia-inducible factor-1α; Low-intensity pulsed ultrasound

Introduction

Angiogenesis is an essential component of skeletal development, and vascular endothelial growth factor (VEGF) signaling plays an important role in this process [1]. VEGF-A is the most abundant of the four biologically active forms and is commonly used in studies investigating the biological effects of VEGF [2]. Exogenous VEGF has been found to enhance blood vessel formation and callus formation of fractured femurs in mice [3]. Targeted deletion of VEGF gene has caused skeletal defect in mice by impairing angiogenesis and endochondral bone formation [4]. During fracture healing, there is cross-talk between endothelial cells and osteoblasts. VEGF has also been found to induce endothelial cell migration, proliferation, and capillary permeability of vascularization process in endochondral bone [5]. These findings indicate that osteoblast-derived VEGF may play an important role in regulating angiogenesis in bone microenvironment during bone formation.

* Correspondence author. Department of Orthopedic Surgery, Chang Gung University, Linkou, Taiwan, 123 Ta-Pei Road, Niao-Sung, Kaohsiung 833, Taiwan.
E-mail address: wangfs@ms33.hinet.net (F.-S. Wang).

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Bone regeneration and blood vessel formation of fractured callus can be promoted by pulsed mechanical stimulation such as low-intensity pulsed ultrasound (US) and extracorporeal shock waves [6–9]. Mechanical stimulation has been found to raise the adaptive modeling response of the bone microenvironment via induction of anabolic or cytokine molecules [10]. Several cell types respond to mechanical stimulation including fluid shear stress and stretch by elevating VEGF mediation of angiogenic responses [11,12]. Previous studies have demonstrated that US could raise the osteogenic response of osteogenic cells through induction of nitric oxide, prostaglandin E_2, and growth factors [13–15]. However, the effect of US stimulation on angiogenic factor production by osteoblasts has not been determined.

Nitric oxide (NO) is produced from a guanidino-nitrogen of L-arginine and dioxygen by three isoforms of nitric oxide synthases (NOS) including constitutive endothelial NOS (eNOS) and neuronal NOS (nNOS) isoform and an inducible form of inducible NOS (iNOS). NO has emerged as a potent regulator for fracture healing, mechanical-stimulated bone formation [16,17], and modulation of VEGF gene transcription in endothelial cells [18,19]. We hypothesized that US treatment may provoke reactive radicals, which in turn may activate the production of angiogenic factor of bone cells.

The purposes of this study were to examine the effect of US on the production of VEGF by human osteoblasts and to investigate whether US promotion of VEGF-A can be linked to the induction of NO and the activation of angiogenic transcription hypoxia-inducible factor-1α (HIF-1α).

Materials and methods

Cell cultures

Human fetal preosteoblastic hFOB 1.19 cells (American Type Culture Center, Manassas, VA, USA) were maintained in a mixture of phenol red-free Ham's F12 medium and DMEM (1:1) containing 10% FBS and 2.5 mM L-glutamine (Life Technologies, Gaithersburg, MD, USA). These cells have osteoblastogenic capacity and can form mineralized nodules under appropriate conditions [20]. Human osteoblastic MG63 and SaOS2 cells were maintained in DMEM containing 10% FBS. Cell cultures were cultured in a 5% CO_2, 37°C incubator for 6 days and were harvested by trypsinization and resuspended in medium for further studies.

Ultrasound treatment

Cells (5×10^5 cells/well, six-well plates) were cultured for 48 h and subjected to US treatment as previously described [13]. Briefly, culture plates were floated in a thermostastically controlled water bath. A UV-sterilized transducer (Sonic Accelerated Fracture Healing System; Exogene, Inc. USA) that generated 1.5 MHz US in a pulsed-wave mode (200 μs pulse burst width with repetition frequency of 1 kHz at the intensity of 30 mW/cm^2) was immersed vertically into each culture well and placed to just touch the surface of the medium. The distance between the transducer and the cells was approximately 5–6 mm. Exposure time was 20 min for all cultures. Control samples were prepared in the same manner, without US exposure. Cells were harvested at 15, 30 min, 1, 3, 6, 12, 24, and 48 h after US stimulation. To elucidate the role of prostaglandin E_2, calcium, and NO in US-promoted VEGF expression, cells were pretreated with or without 10 μM indomethacin (a cyclooxygenase-2 inhibitor) or 100 μM *N*-nitro-L-arginine methyl ester (L-NAME; a pan NOS inhibitor), 100 μM *N*-(3-(aminomethyl)benzyl)acetamidine (1400 W, a highly selective iNOS inhibitor; Calbiochem-Novabiochem GmbH, Schwalbach, Germany) [21], 1 mM EGTA (an extracellular calcium chelator), or 40 μM BAPTA (an intracellular calcium chelator). To investigate whether US promotion of VEGF-A was transcriptionally or translationally regulated, cells were pretreated with 10 μg/ml actinomycin D or 10 μg/ml cyclohexamide (Sigma Inc., St. Louis, MO, USA). To investigate whether guanylate cyclase was involved in US-promoted VEGF-A expression, cells were pretreated with 10 μM ODQ. In some experiments, osteoblasts were cocultured with 20 μM 2,2′-(hydroxynitrosohydrazino)bis-ethanamine (NOC-18) or *S*-nitroso-*N*-acetylpenicillamine (SNAP; Calbiochem-Novabiochem GmbH) for 48 h and subjected to assessment of VEGF-A expression, cell proliferation, and bone alkaline phosphatase activity.

RT-PCR

Total RNA was extracted and purified by the Tri reagent (Sigma Inc.) from 10^6 cells with or without US treatment. One microgram of total RNA was reverse-transcribed (RT) into cDNA, followed by PCR amplification using the following human gene-specific primers: VEGF-A (forward) (5′-TTA TAC CGG GAT TTC TTG CG-3′), (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 previously described [22]. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. All signals were quantified by scanning densitometry, and the final values were obtained by calculating the VEGF-A/β-actin ratios. The fold promotion was calculated as the increase over the value of its corresponding control sample.

VEGF-A levels in the culture supernatants

The VEGF-A levels in culture supernatants were determined using ELISA kits (Quantikine®, R and D Systems

Inc., MN, USA) according to the manufacturer's instructions. Results were calculated by interpolation from a standard curve made by a series of VEGF-A concentrations and normalized with the cell number.

NO production

Nitrite and nitrate levels in culture supernatants were determined using a nitrogen oxide analyzer (NOA280; Sievers Inc., Denver, USA) as previously described [22]. Results were normalized with protein concentration in each sample using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, USA) and were expressed as μM NO/mg protein/min.

Cell proliferation

Cells (5×10^5 cells/well, six-well plate) with or without US treatments were cultured in DMEM with 10% FBS for 12 h before the addition of 1 μCi [^{3}H]-thymidine/well (Amersham-Life Science, Aylesbury, England) for an additional 12-h culture. At the end of the culture period, the cells in each well were released from the plates by trypsinization and processed for [^{3}H]-thymidine uptake determination by liquid scintillation counting (Tri-Crab 2100TR, Packard Inc., USA) [23].

Alkaline phosphatase activity

Cytosolic and nuclear extracts of cell cultures were harvested as previously described [22]. Alkaline phosphatase activities in cytosolic extracts were measured the conversion of *p*-nitrophenyl phosphate into *p*-nitrophenol as previously described [22]. Results were normalized with protein concentration in each sample.

NOS catalytic activities

NOS activities in cytosolic extracts were measured using a NOS activity detection system (Sigma Inc.) according to the manufacturer's instructions. Briefly, aliquots of cytosolic extracts were incubated in a buffer containing 10 μM [^{3}H]-L-arginine, 1 mM NADPH, 5 mM tetrahydrobiopterin, 50 mM valine, 2 mM $CaCl_2$, and 30 nM calmodulin for 30 min at 37°C. Mixtures were centrifuged through spin columns, and [^{3}H]-L-citrulline in eluants was measured using a liquid scintillation analyzer. Parallel experiments were performed in the presence of 2 mM EGTA or 3 mM L-NAME to determine calcium-dependent and -independent NOS activities. Results were normalized with protein concentration in each sample [16].

Cytosolic NOS and nuclear HIF-1α immunoblotting

Cytosolic extracts (500 μg) were immunoprecipitated with monoclonal anti-iNOS, anti-eNOS, anti-nNOS antibodies, and protein A agarose (Upstate Biotechnology, Lake Placid, NY, USA), respectively. Nuclear extracts were immunoprecipitated with monoclonal anti-HIF-1α antibodies (Santa Cruz Biotechnology Inc., CA, USA). Immunoprecipitates were subjected to Western blot assay. The NOSs and HIF-1α bands on the blots were recognized by respective antibodies and horseradish peroxidase-conjugated IgG secondary antibodies and visualized using chemiluminescence agents (SuperSignal®; Pierce Co., Rockford, IL, USA). The phosphorylated HIF-1α bands on the blots 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 monoclonal antiphosphotyrosine 4G10 antibodies (Upstate Biotechnology) using a similar procedure.

Electrophoretic mobility shift assay

An HIF-1 consensus VEGF-A promoter (italicized) oligonucleotide probe (5′-CCA CAG CA*T ACG TGG GC*T CCA ACA-3′, 3′-GGT GTC GT*A TGC ACC CG*A GGT TGT-5′) was 5′ end-labeled with [γ-^{32}P] using T4 polynucleotide kinase and [γ-^{32}P]-ATP (3000 Ci/mmole at 10 mCi/ml; Amersham Pharmacia, Uppsala, Sweden). Nuclear extracts (10 μg) were incubated with a binding buffer containing 10 mM HEPES, pH 7.9, 1 mM DTT, 1 mM EDTA, 80 mM KCl, 20% glycerol and 0.25 mg/ml poly(dI-dC) (Amersham Pharmacia), and 1.75 *p*mole [γ-^{32}P]-labeled oligonucleotide probe (30,000–40,000 cpm; 2μl). To specify protein- or DNA-binding reactions, 1 μl anti-HIF-1α antibodies were added to the binding buffer, incubated for 30 min at 4°C, and mixed with [γ-^{32}P]-labeled oligonucleotide probe. Samples were electrophoresed through a 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 bands were visualized using Kodak Bio-Max film with an intensifying screen at −70°C.

In vitro angiogenesis assay

Concentrated culture supernatants were harvested by lyophilizing 1 ml of culture supernatants of osteoblasts with or without US treatment in the presence or absence of 100 μM L-NAME and then resuspended in 100 μl DMEM. 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 or IgG (R and D Systems Inc.). Concentrated culture supernatants were subjected to assessment of in vitro angiogenesis. Human umbilical vein endothelial cells (1×10^4 cells/well; 48-well plates; American Type Culture Center) were seeded onto a 100-μl three-dimensional matrigel containing DMEM and incubated for 4 h. The human umbilical vein endothelial cells (HUVEC) were treated with concentrated conditioned

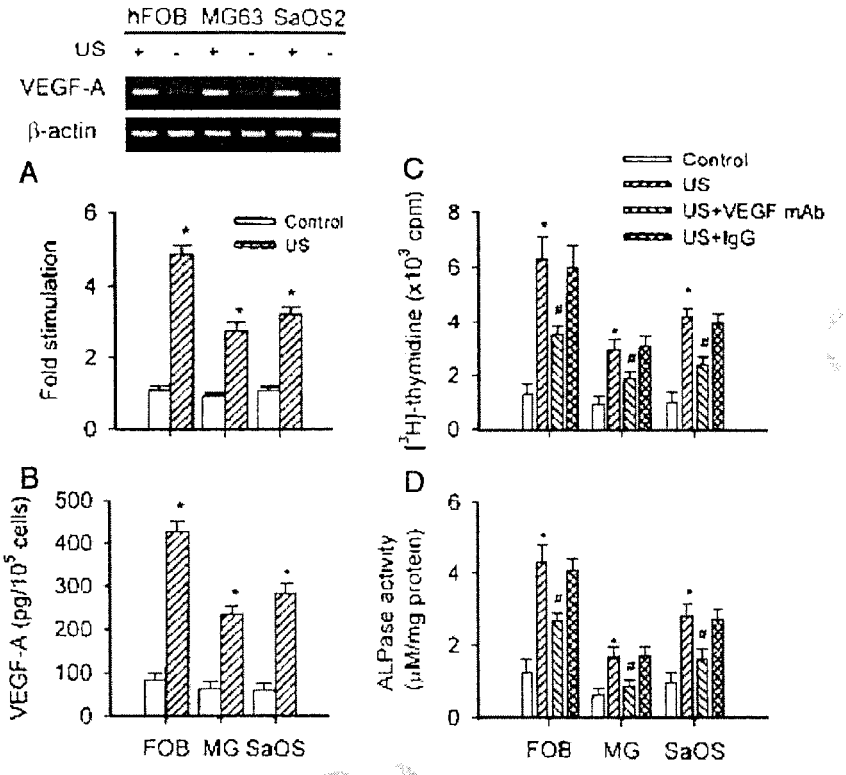

Fig. 1. US promoted VEGF-A (A) mRNA and (B) protein levels. The hFOB1.19, MG63, and SaOS2 osteoblastic cells (5×10^5 cells/well, six-well plates) were treated with US for 20 min. Cells were subjected to assessment of VEGF mRNA 12 h after US treatment. After standardization of housekeeping gene expression, equal amounts of cDNA from each sample were subjected to 40 cycles of PCR to amplify VEGF-A mRNA expression. VEGF-A protein levels in culture supernatants were determined by ELISA assay. US increased (C) [^{3}H]-thymidine uptake in 24 h and (D) bone alkaline phosphatase activities in 48 h. Neutralization with 50 ng/ml monoclonal VEGF-A antibodies partially reduced the promoting effect of US treatment on cell growth and bone alkaline phosphatase activities. Results are presented as mean values ± standard errors calculated from six paired triplicate experiments (* shows difference from control group, # shows difference from US group, $P < 0.05$).

medium and incubated for 6 h to allow tube-like structure formation. Cells were photographed under an inverted microscope, and total network length (defined as an elongation of cells into tube-like structures typically seen in three-dimensional cultures) was quantified in three fields per experiment.

Statistical analysis

All values were expressed as means ± standard errors. Student paired t test was used to evaluate differences between the sample of interest and its respective control. For analysis of time course, a multiple range of ANOVA was used. A P value of <0.05 was considered significant.

Results

US treatment raised VEGF-A mRNA expression

We first determined whether US could augment VEGF-A mRNA expression in human osteoblasts. The

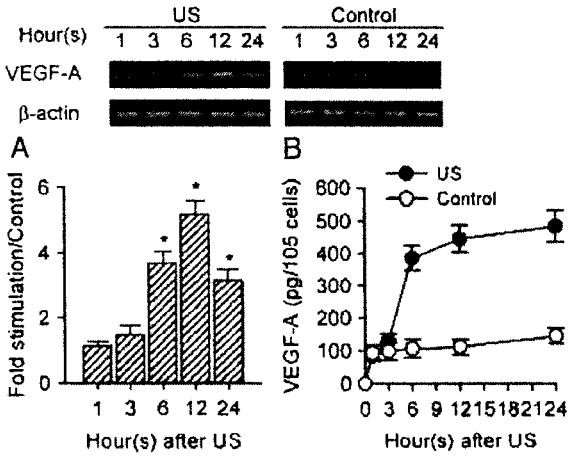

Fig. 2. US promoted VEGF-A (A) mRNA in 6 h and (B) protein levels of osteoblasts in 12 h. The hFOB1.19 osteoblasts (5×10^5 cells/well, six-well plates) were treated with US for 20 min. Cells were subjected to assessment of VEGF mRNA and protein levels 1, 3, 6, 12, and 24 h after US treatment. Results are presented as mean values ± standard errors calculated from six paired triplicate experiments (* shows difference from control group, $P < 0.05$).

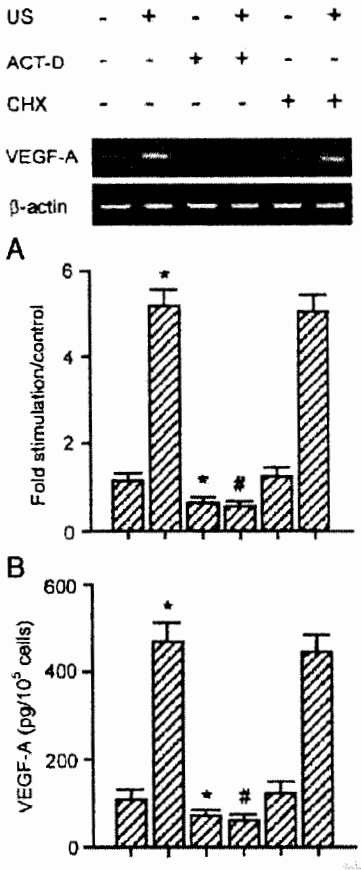

Fig. 3. US promotion of VEGF-A (A) mRNA and (B) protein levels was transcriptionally regulated. The hFOB 1.19 cells underwent 6-h exposure to 10 μg/ml actinomycin D or 10 μg/ml cyclohexamide, with or without US treatment. Cells were subjected to assessment of VEGF mRNA and protein levels 12 h after US stimulation. Results are presented as mean values ± standard errors calculated from six paired triplicate experiments (* shows difference from control group, # shows difference from US group; $P < 0.05$). ACT-D indicates actinomycin D; CHX, cyclohexamide.

hFOB1.19, MG63, and SaOS2 cells were treated with low-intensity pulsed US for 20 min, respectively. RT-PCR and ELISA results indicated that US significantly increased VEGF-A mRNA (Fig. 1A) and protein levels 12 h following US stimulation. Furthermore, US treatment increased [^{3}H]-thymidine uptake in 24 h (Fig. 1C) and bone alkaline phosphatase activity in 48 h (Fig. 1D). Neutralization with 50 ng/ml monoclonal VEGF-A antibodies partially reduced the promoting effect of US treatment on cell growth (Fig. 1C) and alkaline phosphatase activity (Fig. 1D). IgG neutralization did not affect US promotion of cell growth and alkaline phosphatase activity. Of the osteoblasts, hFOB1.19 cells responded best to US stimulation by increasing VEGF-A levels. They were then used in the succeeding experiments. Kinetic results indicated that US increased VEGF-A mRNA (Fig. 2A) and protein levels (Fig. 2B) in 6 h, peaking at 12 h.

US augmentation of VEGF-A mRNA expression is transcription-controlled

We further defined the action mechanism of US-stimulated VEGF-A mRNA expression in cell cultures. Blockade of transcription by 10 μg/ml actinomycin D decreased baseline and US-stimulated VEGF mRNA expression (Fig. 3A) and protein levels (Fig. 3B). Inhibition of translation by 10 μg/ml cyclohexamide did not significantly affect baseline or US-augmented VEGF mRNA (Fig. 3A) and protein levels (Fig. 3B), indicating that US stimulation of VEGF expression is transcriptionally mediated.

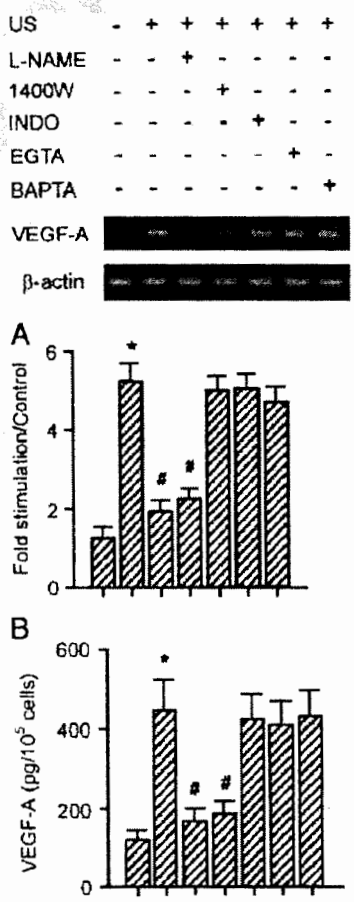

Fig. 4. US-enhanced VEGF-A (A) mRNA and (B) protein levels were mediated by NO but not by PGE_2 or calcium. The hFOB1.19 cells were pretreated with 100 μM 1400W or 100 μM L-NAME or 10 μM indomethacin or 1mM EGTA or 40 μM BAPTA, respectively. Cell cultures were subjected to analysis of VEGF-A expression 12 h after US stimulation. Results are presented as mean values ± standard errors calculated from six paired triplicate experiments (* shows difference from control group, # shows difference from US group; $P < 0.05$).

US promotion of VEGF-A expression was regulated by NO but not by PGE_2 or calcium

We sought to elucidate which mediator was responsible for the increase in VEGF-A expression. Inhibition of cyclooxygenase-2 activity by 10 μM indomethacin and chelating calcium by EGTA or BAPTA did not significantly affect US-promoted VEGF-A mRNA (Fig. 4A) and protein levels (Fig. 4B). Inhibition of NOS activity by 100 μM L-NAME or 100 μM 1400 W significantly reduced US enhancement of VEGF-A mRNA (Fig. 4A) and protein levels (Fig. 4B), suggesting that NO was responsible for mediating US promotion of VEGF-A production.

US induced NOS catalytic activities and iNOS expression

US stimulation significantly increased NO production in 15 min. This increased production of NO persisted for 24 h (Fig. 5A). We further verified which NOS was responsible for US-augmented NO production. US treatment increased calcium-dependent NOS catalytic activity of osteoblasts in 15 min and calcium-independent catalytic activity in 6 h as determined by [^{3}H]-L-arginine conversion to [^{3}H]-L-citrulline (Fig. 5B). Immunoblotting results showed that US treatment induced iNOS expression in 6 h (Fig. 5C). US did not significantly affect eNOS protein expression (Fig. 5C). There was no nNOS immunoreactivity detected throughout the study period (data not

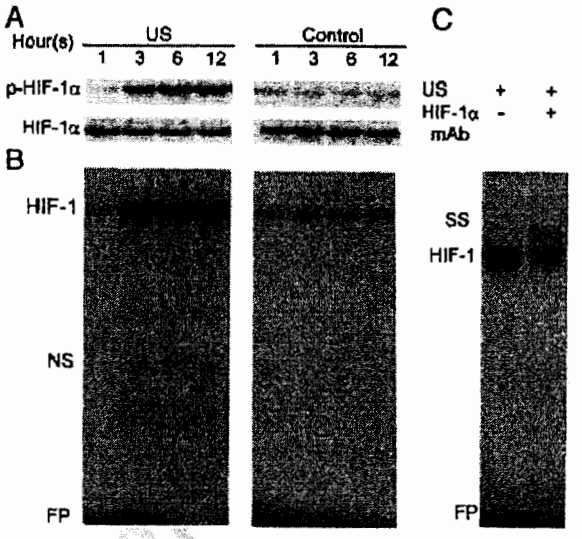

Fig. 6. US activated (A) nuclear HIF-1α phosphorylation in 3 h. Nuclear HIF-1α immunoprecipitates isolated from nuclear extracts of osteoblasts with or without US treatment were subjected to immunoblotting. Phosphorylated HIF-1α was probed using antiphosphotyrosine antibodies. (B) US promoted HIF-1α binding to the VEGF-A promoter in 3 h as determined by gel shift assay. (C) Autoradiograph of HIF-1α supershift. Nuclear extracts from osteoblasts with or without US stimulation were incubated with HIF-1 probe in the presence or absence of anti-HIF-1α antibodies. All blots and autoradiographs are representative of at least three different experiments with comparable results. FP indicates free probe; SS, supershift.

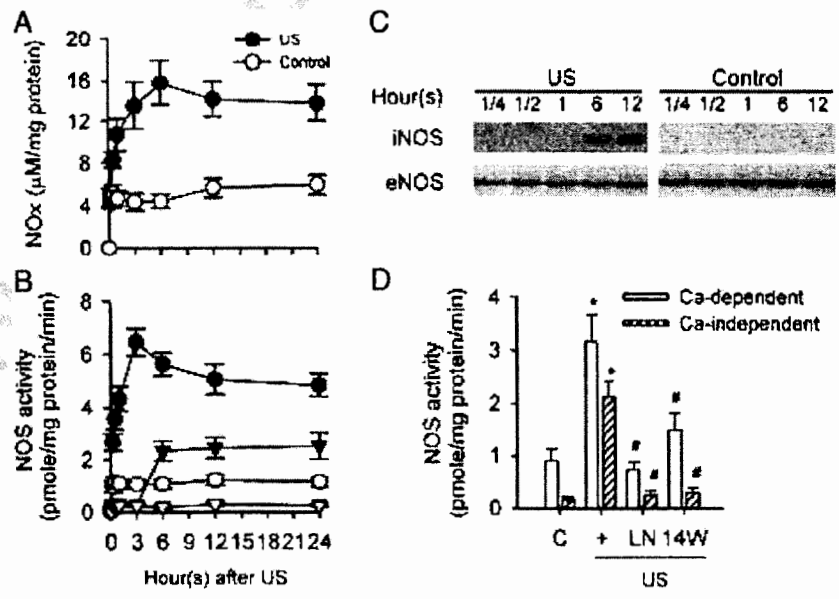

Fig. 5. US increased (A) NO production in 15 min, (B) calcium-dependent NOS activity in 15 min, and calcium-independent NOS activity in 6 h. (C) US treatment rapidly induced iNOS expression in 6 h. US stimulation did not significantly affect eNOS expression throughout the study period. All blots are representative of at least three different experiments with comparable results. (D) L-NAME or 1400 W pretreatment reduced US promotion of calcium-dependent NOS activity in 15 min and calcium-independent NOS activity in 6 h. Results are presented as mean values ± standard errors calculated from six paired triplicate experiments (* shows difference from control group, # shows difference from US group; $P< 0.05$). Symbols: ● indicates calcium-dependent NOS activity in US groups; ○, calcium-dependent NOS activity in control groups; ▼, calcium-independent NOS activity in US groups; ▽, calcium-independent NOS activity in control groups; C, control; LN, L-NAME; 14 W, 1400 W.

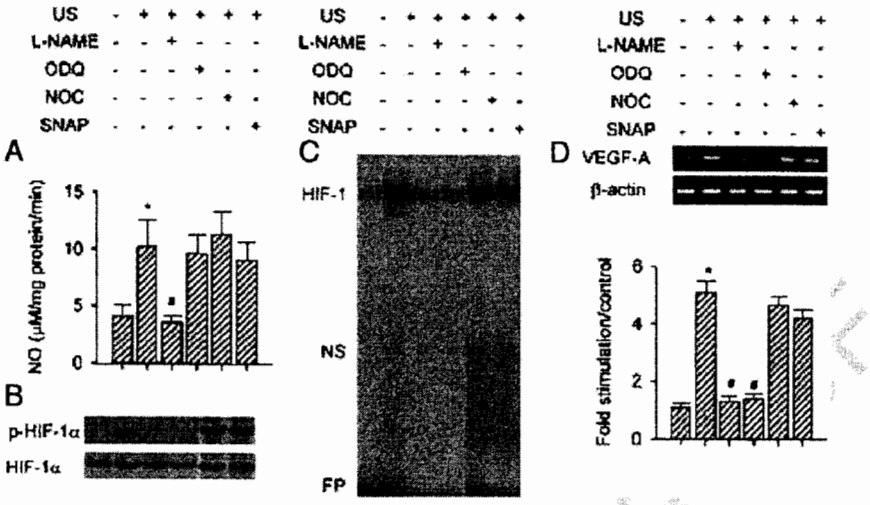

Fig. 7. (A) L-NAME attenuated US-induced NO production in 1 h. (B) 100 μM L-NAME or 5 μM ODQ pretreatment attenuated US-enhanced nuclear HIF-1α phosphorylation in 3 h, (C) HIF-1α binding to the VEGF-A promoter in 3 h, and (D) VEGF-A mRNA expression in 12 h. NO donors (30 μM NOC-18 or SNAP) mimicked the promoting effect of US treatment on (B and C) HIF-1α activation and (D) VEGF-A mRNA expression. Results are presented as mean values ± standard errors calculated from six paired triplicate experiments (* shows difference from control group, # shows difference from US group; $P < 0.05$). All blots and autoradiographs are representative of at least three different experiments with comparable results.

shown). L-NAME and 1400 W pretreatment significantly reduced US-promoted calcium-dependent NOS activity in 15 min and calcium-independent NOS activity in 6 h (Fig. 5D), respectively.

US promoted HIF-1α transactivation

Osteoblasts subjected to US treatment increased nuclear HIF-1α phosphorylation, as demonstrated by phosphotyrosine expression of HIF-1α in 3 h (Fig. 6A), and promoted HIF-1 binding to the VEGF-A promoter, as determined by electrophoretic gel shift (Fig. 6B). We employed antibodies against HIF-1α to confirm the DNA-protein-binding activity. Autoradiograph showed that nuclear extract harvested from US-treated osteoblasts was indeed supershifted by anti-HIF-1α antibodies (Fig. 6C), indicating that US treatment did activate HIF-1α binding to the VEGF-A promoter.

L-NAME and ODQ pretreatment reduced US-augmented HIF-1α activation and VEGF expression

We employed L-NAME and ODQ to determine the roles of NOS and guanylate cyclase in US-stimulated VEGF expression of osteoblasts. Inhibition of NOS activity significantly reduced US-promoted NO production in 1 h (Fig. 7A). Pretreatment with L-NAME or ODQ decreased US-activated nuclear HIF-1α phosphorylation (Fig. 7B), HIF-1α binding to the VEGF-A promoter in 3 h (Fig. 7C), and VEGF-A mRNA expression in 12 h (Fig. 7D). Blockade of NOS or guanylate cyclase activity significantly reduced US promotion of VEGF-A protein levels, cell proliferation, and

t1.1 Table 1
t1.2 Effect of NOS inhibitors, guanylate cyclase inhibitor, and NO donors on VEGF-A expression, cell proliferation, and alkaline phosphatase activities of human osteoblasts with or without US stimulation

t1.3		Cells	Control	Ultrasound stimulation				NOC-18	SNAP
t1.4				Vehicle	L-NAME	1400W	ODQ		
t1.5	VEGF-A (pg/10^5 cells)	hFOB1.19	92 ± 16	428 ± 42*	143 ± 19**	196 ± 28**	178 ± 22**	376 ± 39*	358 ± 25*
t1.6		MG63	85 ± 17	252 ± 26*	112 ± 21**	102 ± 19**	109 ± 15**	228 ± 24*	232 ± 21*
t1.7		SaOS2	91 ± 17	302 ± 27*	125 ± 18**	116 ± 14**	121 ± 17**	263 ± 19*	274 ± 20*
t1.8	[^{3}H]-thymidine uptake	hFOB1.19	1.38 ± 0.31	6.28 ± 0.87*	1.52 ± 0.20**	1.74 ± 0.24**	1.62 ± 0.32**	1.21 ± 0.28	1.15 ± 0.22
t1.9	(× 10^3 cpm)	MG63	0.94 ± 0.23	3.14 ± 0.40*	1.18 ± 0.19**	1.22 ± 0.27**	1.19 ± 0.33**	1.04 ± 0.17	0.91 ± 0.16
t1.10		SaOS2	1.08 ± 0.28	4.21 ± 0.38*	1.25 ± 0.23**	1.38 ± 0.30**	1.45 ± 0.29**	1.13 ± 0.20	1.07 ± 0.15
t1.11	ALPase activity	hFOB1.19	1.12 ± 0.19	4.21 ± 0.48*	1.67 ± 0.25**	1.98 ± 0.23**	1.73 ± 0.21**	1.21 ± 0.18	1.15 ± 0.23
t1.12	(μM/mg protein/min)	MG63	0.86 ± 0.14	1.82 ± 0.28*	1.08 ± 0.19**	1.15 ± 0.26**	1.04 ± 0.19**	0.79 ± 0.17	0.90 ± 0.19
t1.13		SaOS2	1.04 ± 0.16	2.87 ± 0.21*	1.14 ± 0.16**	0.92 ± 0.23**	1.12 ± 0.14**	1.12 ± 0.21	0.94 ± 0.17

t1.14 Results are presented as mean values ± standard errors calculated from six triplicate experiments.
t1.15 *Shows difference from control group, $P < 0.05$.
t1.16 **Shows difference from vehicle group; $P < 0.05$.

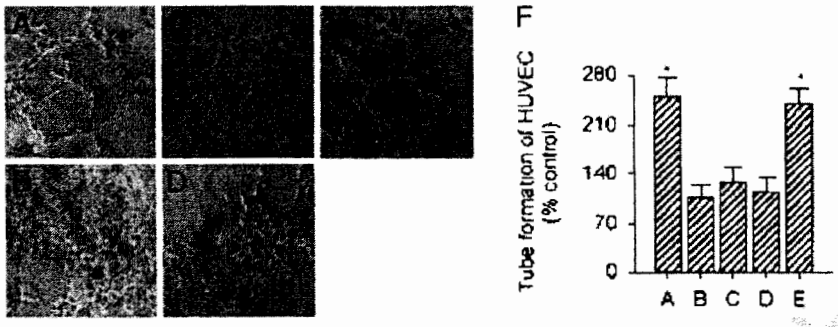

Fig. 8. Culture supernatants obtained from (A) US-treated osteoblasts promoted tube formation of HUVEC in comparison with the (B) control without US treatment. (C) Neutralization of VEGF-A with monoclonal VEGF-A antibody reduced promoting effect of culture supernatants on angiogenesis. (D) Inhibition of iNOS activity by L-NAME decreased angiogenic responses. (E) IgG neutralization did not affect US-promoted angiogenesis. (F) Results are presented as mean values ± standard errors calculated from six paired triplicate experiments (* shows difference from B group; $P < 0.05$).

bone alkaline phosphatase activities (Table 1). These results suggested that NO mediated US promotion of VEGF-A expression through cGMP-dependent HIF-1α activation. Cell cultures were treated with NO donors to mimic US promotion of VEGF expression. NOC-18 and SNAP promoted HIF-1α activation (Figs. 7B and C) and VEGF-A mRNA (Fig. 7D). NO donors increased VEGF-A protein level but not cell growth or bone alkaline phosphatase activities (Table 1).

US stimulated in vitro angiogenesis through NO-mediated VEGF-A

We tested whether US could induce in vitro angiogenesis through NO-mediated VEGF-A. Culture supernatants from US-treated osteoblasts were found to significantly increase tube formation of HUVEC (Fig. 8A) when compared with the control without US treatment (Fig. 8B). Monoclonal VEGF-A antibody neutralization (Fig. 8C) or L-NAME inhibition of NO production (Fig. 8D) significantly reduced the promoting effect of culture supernatant on tube formation. Neutralization with IgG did not alter the promoting effect of conditioned medium from US-treated cell cultures on angiogenesis of HUVEC (Fig. 8E). Effect of conditioned medium on tube formation of HUVEC was measured (Fig. 8F).

Discussion

Elevations of VEGF-A mRNA and protein levels occurred following US stimulation, which rapidly induced NO production and activated HIF-1α transactivation. Mechanisms underlying US-augmented bone repair are not well understood. Fracture healing is proceeded by vascular invasion, and osteogenesis takes place near neovessels that mediate delivery of osteoprogenitors, secrete mitogens for osteoblasts, and transport nutrients and oxygen [24]. Our findings provide the first indication that osteoblasts responded to US by increasing NO-mediated VEGF-A induction. We provide clear evidence that US stimulation can be an effective biophysical method for enhancing osteogenic activities and angiogenic factor production by osteoblasts. The raise in angiogenic factor production by osteoblasts may further explain the clinical success of US treatment on promotion of fracture healing.

In our study, [^{3}H]-thymidine uptake and bone alkaline phosphatase activities of osteoblasts were increased following US stimulation. Monoclonal VEGF-A antibody neutralization attenuated the promoting effect of US treatment on osteogenic activities and tube formation of HUVEC. These data suggest that osteoblasts could convert acoustic energy released by physical US stimulation into mitogenic signals and the production of an angiogenic factor. VEGF-A is involved in regulating US-promoted growth and differentiation of osteoblasts. Our findings support previous studies in which VEGF was found to elicit proliferative and chemotactic effects on osteoblasts [25,26]. Angiogenesis is considered essential for endochondral ossification during proper fracture healing. Administration of an angiogenesis inhibitor has been found to impair fracture healing [27]. VEGF is important for normal endochondral bone development to not only mediate bone vascularization, but also allow normal differentiation of hypertrophic chondrocytes and osteoblasts. Strong VEGF expression of osteoprogenitors has been found to occur in the early stage of fracture healing [28]. Blockade of VEGF activity using a specific antagonist (soluble Flt) inhibited bone callus formation [29]. Moreover, acceleration of fracture healing has been found in experimental animals after VEGF-A gene transfer and recombinant VEGF-A protein treatment [3,30], indicating that VEGF plays a crucial role in regulating osteochondrogenesis and angiogenesis during fracture healing. In the present study, pretreatment with L-NAME or ODQ reduced US promotion of osteogenic activity and VEGF-A synthesis. These findings suggest that NO-mediated cGMP was involved in regulating US-promoted osteogensesis and angiogenic factor production by osteoblasts. While NO donors

mimicked the promoting effect of US on VEGF-A production, NO donors did not alter cell proliferation and bone alkaline phosphatase activity of osteoblasts. We speculate the differences that multiple signal transduction pathways may be involved in US-promoted osteogenic activity and angiogenic factor production by osteoblasts.

In contrast to previous studies demonstrating that calcium and prostaglandin E_2 were involved in regulating the anabolic responses of fluid flow-stimulated osteoblasts [31,32], we showed that NO, but not PGE_2 or calcium, acted as a critical mediator to communicate biophysical US stimuli into intracellular responses that caused synthesis of a factor capable of initiating angiogenic responses. We speculate that bioactive mediators involving mechanical stimulation of osteoblasts may depend on the model system, mechanical stimuli type, and cell type used. A previous study suggested that pulsed fluid flow-increased NO production by human bone cell cultures was eNOS-dependent [33]. Reher et al. [13] have demonstrated that US increases in NO synthesis by osteoblasts are regulated by iNOS. In the current study, US promotion of VEGF production was via transcriptional regulation, indicating that US augmentation of VEGF levels did not require the synthesis of intermediary proteins. While later elevation of iNOS expression and calcium-independent NOS activity were noted in osteoblasts following US stimulation, L-NAME (a pan NOS inhibitor) or 1400 W (a highly selective iNOS inhibitor) pretreatment reduced US promotion of calcium-dependent NOS catalytic activities and VEGF expression. These findings suggest that early activation of calcium-dependent NOS catalytic activity is required for US promotion of VEGF synthesis. Moreover, NO donor treatments also elicited promoting effects on VEGF production. Our findings suggest that eNOS plays an important role in regulating US promotion of angiogenic factor production by osteoblasts. We failed to detect nNOS immunoreactivity throughout the study period. Our investigations on detection of nNOS immunoexpression and NO donor stimulation of cell proliferation and alkaline phosphatase activity are in agreement with a study by MacPherson et al. [34].

In the current study, we showed that osteoblasts responded to US stimulation by activating HIF-1α (a VEGF transcription factor) binding to the VEGF-A promoter and VEGF-A mRNA levels. A growing body of evidence has suggested that NO is involved in regulating VEGF expression through activation of HIF [35,36]. HIF-1 is a heterodimeric basic helix–loop–helix transcription factor and acts as a critical regulator for VEGF expression. Active HIF-1 has been reported to accumulate in the cell nucleus, bind to the target DNA sequence, and enhance VEGF gene transcription [37]. We suggest that HIF-1α is likely a target of mechanical signal by which physical US stimulation dictates the cellular and angiogenesis-promoting activities of osteoblasts. It is not presently known what molecule may be responsible for regulating VEGF expression in osteoblasts after US stimulation. NO has been found to mediate HIF-1α stabilization, nuclear translocation, and activation in mechanical-stressed myocardiac cells [38]. In this study, we noted that inhibition of NOS or guanylate cyclase activity reduced the promoting effect of US stimulation on HIF-1α transactivation and VEGF-A mRNA expression. These findings indicate that NO through cGMP-dependent pathway mediates US promotion of cell proliferation and VEGF production by osteoblasts.

The mechanism by which US treatment induces NOS catalytic activities has not been clarified. Bone cells perceive and respond to mechanical forces via mechanotransduction pathways. Mechanoreceptors convert biophysical stimuli into biochemical responses that alter gene expression and cellular adaptation. One of our recent studies has demonstrated that pertussis toxin-sensitive Gα1 proteins mediates US activation of osteogenic transcription (Cbfa1/Runx2) and increases osteocalcin gene expression in hFOB1.19 osteoblasts [39]. Our findings imply that US-sensitive biomolecules located at the cell surface may be activated by US to induce eNOS catalytic activity and iNOS expression. Further studies are needed to explore the role of membrane-bound mechanosensitive molecules, such as G proteins, exracellular integrins, and ion channels, in regulating US-induced angiogenic factor production by osteoblasts. Taken together, we provide evidence that osteoblasts rapidly respond to increase NO production and angiogenic gene expression after US stimulation. Results from these studies suggest that regulation of reactive nitrogen reactions by biophysical factors such as US might provide a promising regimen for the regulation of angiogenic transcription factor, HIF-1α, resulting in increase of VEGF-A production.

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