THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 35, pp. 25406–25415, August 31, 2007

Ultrasound Induces Hypoxia-inducible Factor-1 Activation and Inducible Nitric-oxide Synthase Expression through the Integrin/Integrin-linked Kinase/Akt/Mammalian Target of Rapamycin Pathway in Osteoblasts*[S]

Received for publication, February 2, 2007, and in revised form, June 19, 2007 Published, JBC Papers in Press, June 21, 2007, DOI 10.1074/jbc.M701001200

Chih-Hsin Tang[‡], Dah-Yuu Lu[§], Tzu-Wei Tan[‡], Wen-Mei Fu[§1], and Rong-Sen Yang[¶2]

From the [‡]Department of Pharmacology, College of Medicine, China Medical University, Taichung 404 and the Departments of [§]Pharmacology and [¶]Orthopaedics, College of Medicine, National Taiwan University, No. 7, Chung-Shan South Road, Taipei 100, Taiwan

It has been shown that ultrasound (US) stimulation accelerates fracture healing in the animal models and clinical studies. Nitric oxide (NO) is a crucial early mediator in mechanically induced bone formation. Here we found that US stimulation increased NO formation and the protein level of inducible nitric-oxide synthase (iNOS). US-mediated iNOS expression was attenuated by anti-integrin $\alpha5\beta1$ or $\beta1$ antibodies but not anti-integrin $\alpha v\beta3$ or $\beta3$ antibodies or focal adhesion kinase mutant. Integrin-linked kinase (ILK) inhibitor (KP-392), Akt inhibitor (1*L*-6-hydroxymethyl-*chiro*-inositol-2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate]) or mammalian target of rapamycin (mTOR) inhibitor (rapamycin) also inhibited the potentiating action of US. US stimulation increased the kinase activity of ILK and phosphorylation of Akt and mTOR. Furthermore, US stimulation also increased the stability and activity of HIF-1 protein. The binding of HIF-1α to the HRE elements on the iNOS promoter was enhanced by US stimulation. Moreover, the use of pharmacological inhibitors or genetic inhibition revealed that both ILK/Akt and mTOR signaling pathway were potentially required for US-induced HIF-1α activation and subsequent iNOS up-regulation. Taken together, our results provide evidence that US stimulation up-regulates iNOS expression in osteoblasts by an HIF-1α-dependent mechanism involving the activation of ILK/Akt and mTOR pathways via integrin receptor.

Fracture healing is a complex physiological process comprising the coordinated participation of several cell types. Among all the means to influence fracture healing, ultrasound (US)[3] distinguishes itself by being non-invasive and easy to apply. Low intensity levels are used to accelerate fracture healing and are considered neither thermal nor destructive. It has been shown that low intensity US accelerates fracture healing in animal models (1, 2) and clinical studies (3, 4). It has been demonstrated that low intensity-pulsed US exposure increases nitric oxide (NO) and prostaglandin release (5, 6), stimulates collagen synthesis, and promotes bone formation (7). However, the mechanisms involved in osteoblasts to detect US stress and transduce the signal across the membrane for activating signaling pathways in metabolism, such as the induction of inducible nitric-oxide synthase (iNOS) and release of NO, remain poorly understood.

Integrins are cell-surface adhesion receptors that regulate cell viability in response to cues derived from the extracellular matrix (8, 9). In the case of mesenchymal cells, encompassed by the extracellular matrix, matrix-derived mechanical stimuli can regulate their viability. In this latter scenario, integrins function as mechanoreceptors that detect mechanical stimuli originating from the extracellular matrix and convert them to chemical signaling pathways that regulate cell viability (10, 11). Mechanical stimuli can be transmitted through the direct or indirect interaction of integrins with associated lipid or protein-signaling molecules in the focal adhesion complex (12, 13). Integrin-linked kinase (ILK), a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling (14). ILK can interact with the cytoplasmic domain of β-integrin subunits and is activated by both integrin activation as well as growth factors and is an upstream regulator of Akt (15).

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor composed of the basic helix-loop-helix-Per-Arnt-Sim-domain, containing the proteins HIF-1α and arylhydrocarbon receptor nuclear translocator (HIF-1β) (16). The availability of HIF-1 is determined primarily by HIF-1α, which is regulated at the protein level in an oxygen-sensitive manner, in contrast to HIF-1β, which is stably expressed (17, 18). During normoxia, HIF-1α is efficiently degraded through the von Hippel-Lindau-dependent ubiquitin-proteasome pathway (18). Under

* This work was supported by the National Science Council of Taiwan (Grant NSC 95-2314-B-039-045) and China Medical University (Grant CMU 95-208). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

[1] To whom correspondence may be addressed: Tel.: 886-2-231-23456 (ext. 8319); Fax: 886-2-23417930; E-mail: wenmei@ntu.edu.tw.

[2] To whom correspondence may be addressed: Tel.: 886-2-231-23456 (ext. 3958); Fax: 886-2-239-36577; E-mail: rsyang@ntuh.gov.tw.

[3] The abbreviations used are: US, ultrasound; iNOS, inducible nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; FAK, focal adhesion kinase; ILK, integrin-linked kinase; mTOR, mammalian target of rapamycin; HIF, hypoxia-inducible factor; GSK3β, glycogen synthase kinase 3β; siRNA, interference RNA; ChIP, chromatin immunoprecipitation; HRE, hypoxia response element.

hypoxia, HIF-1α protein is markedly stabilized, translocates to the nucleus, and heterodimerizes with HIF-1β. The HIF-1α·HIF-1β complex can then bind to hypoxia response elements (HREs) located in gene promoters to regulate transcription of vascular endothelial growth factor, erythropoietin, iNOS, and glycolytic enzymes that enhance cellular adaptation to hypoxia (19).

Intracellular signals that promote osteoblast differentiation, including those mediated by bioactive radicals such us NO, prostaglandin, and calcium, may occur in response to cellular homeostatic disturbance induced by US (5, 20). It has been reported that US exposure increased NO and prostaglandin E_2 release via up-regulation of iNOS and COX-2 in osteoblasts (5). In addition, NO may play an important role in the maturation of osteoblast and differentiation of osteoclast (21). However, the signaling pathways for US stimulation on iNOS expression and bone formation are mostly unknown. Here we found that US stimulation increased iNOS expression and promoted NO production in MC3T3-E1 cells in a HIF-dependent manner involving the activation ILK/Akt/mTOR through the β1 integrin receptor.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antibody for ILK was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies specific for mammalian target of rapamycin (mTOR), phospho-mTOR (Ser-2448), glycogen synthase kinase 3β (GSK3β), and phospho-GSK3β were purchased from Cell Signaling Technology. Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for iNOS, eNOS, phospho-Akt (Ser-473), Akt, HIF-1α, HIF-1β, α-tubulin, and ILK small interference RNA (siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for β1 (mAb1984, Clone BMA5) and α5β1 (mAb2514, Clone BMB5) integrin were purchased from Chemicon. Hamster monoclonal antibodies specific for CD51 (αvβ3 integrin (Clone H9.2B)) and CD61 (β3 integrin (Clone 2C9.2G2, HMβ3-1)) were purchased from BD Biosciences. Both these antibodies have been described as function-blocking antibodies for murine integrin subunits (40). Control isotype antibody was used as negative controls. A selective αvβ3 integrin antagonist cyclic RGD (cyclo-RGDfV) peptide and the cyclic RAD (cyclo-RADfV) peptide were purchased from Peptides International (Louisville, KY) (41). KP-392 was purchased from Kinetek Pharmaceuticals (22). The iNOS promoter construct (piNOS-Luc) was a gift from Dr. E. A. Ratovitski (Johns Hopkins University). pHRE-luciferase construct was a gift from Dr. M. L. Kuo (National Taiwan University, Taiwan). The autophosphorylation site mutant FAK(Y397F) was a gift from Dr. J. A. Girault (Institut du Fer à Moulin, France). The Akt (Akt K179A) dominant negative mutant was a gift from Dr. R. H. Chen (Institute of Molecular Medicine, National Taiwan University, Taiwan). The HIF-1α dominant negative mutant was purchased from American Type Culture Collection (Manassas, VA). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, WI). All other chemicals were obtained from Sigma-Aldrich.

Cell Cultures—A murine osteoblastic cell line MC3T3-E1 was obtained from Riken Cell Bank (Tsukuba, Japan). Cells were grown on the plastic cell culture dishes in 95% air 5% CO_2 with α-minimal essential medium (Invitrogen), which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (pH adjusted to 7.6). The culture medium was changed twice per week.

Ultrasound Treatment—Cells (3×10^5 cells/well, 6-well plates) were cultured for 24 h and subjected to US treatment. A UV-sterilized unfocused circular transducer (Exogen®, Smith & Nephew Inc., Memphis, TN) was immersed vertically into each culture well and placed to just contact the surface of the culture medium. The transducer generated a low intensity-pulsed ultrasound signal that has been clinically proven to enhance fracture healing (3). The driving signal consists of a 1.5-MHz sinusoidal ultrasound carrier wave, amplitude modulated with a 1-kHz pulse, and a pulse width of 200 μs (20% duty cycle). The spatial average time average intensity was 30 milliwatts/cm^2 with a temporal average power of 117 milliwatts and an effective radiating area of 3.88 cm^2. Exposure time was 20 min for all cultures, which is the FDA-approved treatment time for bone healing. The distance between the transducer and the cells was ~5 mm (supplemental Fig. S1). Control samples were prepared in the same manner without US exposure. Cells were harvested at 5, 15, 30, 60, and 120 min after US stimulation (6). For examination of the downstream signaling pathways involved in US treatment, osteoblasts were pretreated with various antibodies (control IgG antibody for comparison) or inhibitors (Me_2SO as vehicle) for 30 min before US stimulation.

Assay of NO—MC3T3-E1 cells (3×10^5 cells in 1 ml/well) were exposed to US in 6-well plates. Production of NO was assayed by measuring the stable metabolite of nitrite levels in the culture medium. Sample aliquots (100 μl) were mixed with 100 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2% phosphoric acid) in 96-well plate and incubated at 25 °C for 10 min. The absorbance at 550 nm was measured on a microplate reader.

Western Blot Analysis—The cellular lysates were prepared as described previously (23). Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membranes. The blots were blocked with 4% bovine serum albumin for 1 h at room temperature and then probed with rabbit anti-mouse antibodies against iNOS, HIF-1α, HIF-1β, p-Akt, or p-mTOR (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuaNT software (Amersham Biosciences).

For the study of HIF-1α translocation, cells were rinsed with phosphate-buffered saline and suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and vortexed for 10 s. The lysates were separated into cytosolic and nuclear fractions by centrifugation at

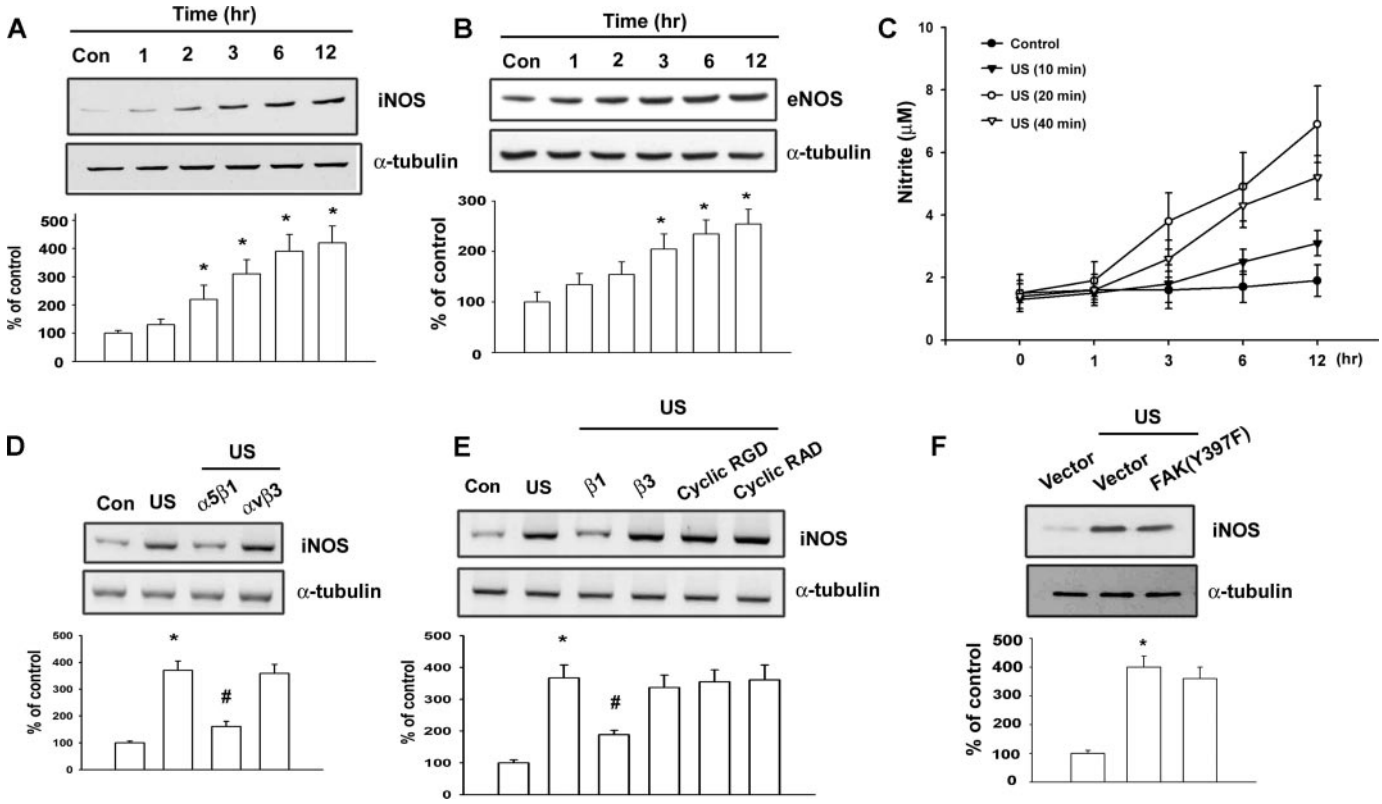

FIGURE 1. **Increase of iNOS expression by US stimulation in cultured osteoblasts.** *A* and *B*, MC3T3-E1 cells were exposed to US for 20 min. Protein levels of iNOS or eNOS were measured 1, 2, 3, 6, and 12 h after US treatment. The quantitative data are shown in the *lower panels* ($n = 4$). *C*, cells were exposed to US for 10, 20, or 40 min. The cultured media were collected at various time intervals after US stimulation. The production of NO was evaluated by Griess reaction by measuring the level of nitrite. Osteoblasts were pretreated with mAb against $\alpha v\beta 3$ or $\alpha 5\beta 1$ integrin (20 μg/ml) (*D*), mAb against $\beta 1$ or $\beta 3$ integrin (20 μg/ml) and cyclic RGD peptide or cyclic RAD (30 μM) (*E*) for 30 min, or transfected with FAK(Y397F) mutant for 24 h (*F*) followed by exposure to US for 20 min. iNOS protein level was determined by immunoblotting at 12 h after US. The quantitative data are shown in the *lower panels* ($n = 4$). Results are expressed as the mean ± S.E. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with US treatment alone.

12,000 × *g* for 15 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in buffer C (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatants containing nuclei proteins were collected by centrifugation at 12,000 × *g* for 15 min and stored at −70 °C. All protein concentration was determined by colorimetric assay using Bio-Rad assay kit (Bio-Rad, Hercules, CA).

Equal amounts (50 μg) of each protein from cytosolic or nuclei fractions were separated by 8% polyacrylamide-SDS gel and then electrotransferred to polyvinylidene difluoride membranes. The blocked membranes were incubated overnight at room temperature with mouse anti-HIF-1α antibody. After washing with phosphate-buffered saline, the blots were incubated for 1 h at room temperature with secondary antibody.

ILK Kinase Assay—ILK enzymatic activity was assayed in MC3T3-E1 cells lysed in Nonidet P-40 buffer (0.5% sodium-deoxycholate, 1% Nonidet P-40, 50 mM HEPES (pH 7.4), 150 mM NaCl) as previously reported (22). Briefly, ILK was immunoprecipitated with ILK antibody overnight at 4 °C from 250 μg of lysate. After immunoprecipitation, beads were resuspended in 30 μl of kinase buffer containing 1 μg of recombinant substrate (GSK3β fusion protein) and 200 μM cold ATP, and the reaction was carried out for 30 min at 30 °C. The phosphorylated substrate was visualized by Western blot with phospho-GSK3β antibody. Total GSK3β was detected with the appropriate antibody.

mRNA Analysis by Reverse Transcriptase-PCR—Total RNA was extracted from osteoblasts using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse-transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide primers: mouse HIF-1α, ATGGAGGGCGCCGGC and GATGATGATATCGCCGCGCT; mouse HIF-1β, TGGGTCATCTTCTCGCGGTT and TGTCCTATCTGAGCATCGTG. Each PCR cycle was carried out for 30 s at 94 °C, 30 s at 55 °C, and 1 min at 68 °C. PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide.

Transfection and Reporter Gene Assay—Osteoblasts were co-transfected with 0.5 μg of iNOS promoter plasmid and 0.5 μg of β-galactosidase expression vector. Osteoblasts were grown to 70% confluent in 6-well plates and were transfected on the following day by Lipofectamine 2000 (LF2000), premix DNA with Opti-MEM, and LF2000 with Opti-MEM, respectively, for 5 min. The mixture was then incubated for 25 min at room temperature and added to each well. After 24-h incubation, transfection was complete, and cells were incubated with the indicated agents. The media were removed 24 h after US stimulation, and cells were washed once with cold phosphate-buffered saline. To prepare lysates, 100 μl of reporter lysis

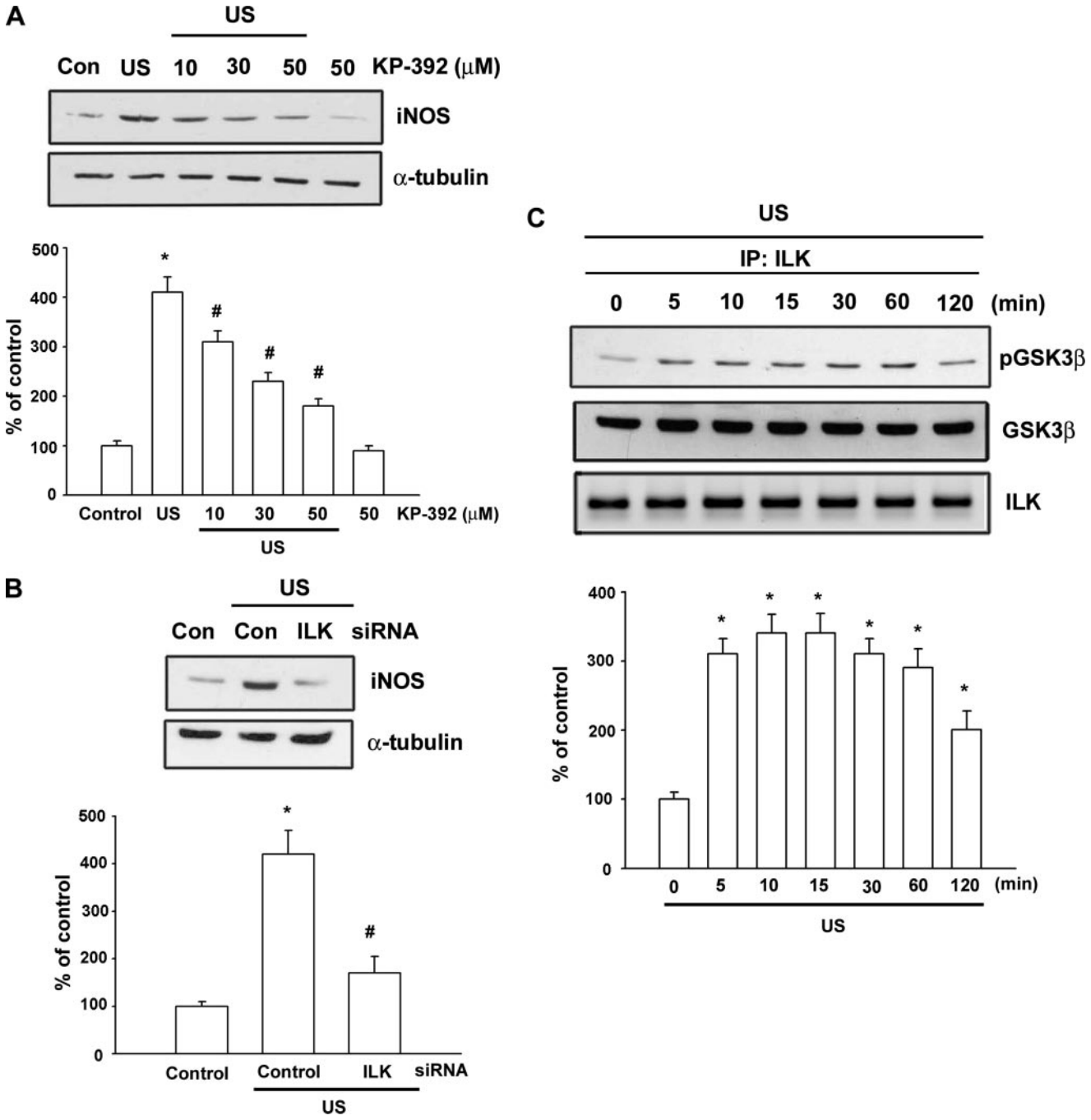

FIGURE 2. **Involvement of ILK signaling pathway in response to US stimulation in osteoblasts.** Osteoblasts were pretreated with ILK inhibitor of KP-392 (10–50 μM) for 30 min (*A*) or transfected with ILK small interference (siRNA) (*B*) for 24 h followed by stimulation with US for 20 min, and iNOS protein level was determined by immunoblotting 12 h following US. The quantitative data are shown in the *lower panels* ($n = 4$). *C*, osteoblasts were stimulated with US for 20 min, and cell lysates were immunoprecipitated (*IP*) with an antibody specific for ILK. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-pGSK3β or GSK3β at various time intervals after US stimulation. The quantitative data are shown in the *lower panels* ($n = 4$). Results are expressed as the mean ± S.E. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with US treatment alone.

buffer (Promega) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 30 s. Aliquots of cell lysates (10 μl) containing equal amounts of protein (10–20 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector. In experiments using dominant-negative mutants, cells were co-transfected with reporter (0.5 μg) and β-galactosidase (0.25 μg) and either the Akt or HIF-1α mutant or the empty vector (0.5 μg).

DNA Affinity Protein-binding Assay—The biotinylated double-stranded oligonucleotides corresponding to the HRE sequences (5′-GTGACTACGTGCTGCCTAG-biotin-3′) of mouse iNOS promoter were synthesized and annealed (24). Binding of transcription factors to the iNOS promoter DNA sequences was assayed, as described (25). Following exposure

to US, nuclear extracts were prepared. Biotin-labeled double-stranded oligonucleotides (2 μg) were mixed at room temperature for 1 h by shaking with 200 μg of nuclear extract proteins, and 20 μl of streptavidin-agarose beads in a 70% slurry. Beads were pelleted and washed three times with cold phosphate-buffered saline, and then the bound proteins separated by SDS-PAGE, followed by Western blot analysis with specific antibodies.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (6). DNA immunoprecipitated by anti-HIF-1α antibody was purified. The DNA was then extracted with phenol-chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized by UV. The primers, 5′-CTGC-CCAAGCTGACTTACTAC-3′ and 5′-GACCCTGGCAG-CAGCCATCAG-3′, were utilized to amplify across the iNOS promoter region (26).

Statistics—The values given are means ± S.E. The significance of difference between the experimental groups and controls was assessed by Student's *t* test. The difference is significant if the *p* value is <0.05.

RESULTS

Effect of Ultrasound Stimulation on iNOS Expression in Osteoblasts—It has been demonstrated that pulse application of low intensity US increased NO release via up-regulation of iNOS (5), which is important for mechanically induced bone formation (27). We then investigated the effect of US stimulation on the iNOS expression in osteoblasts. MC3T3-E1 cells were exposed to US for 20 min, and the cell lysates were collected at different time intervals. The results from Western blot analysis indicated that US time-dependently increased protein levels of iNOS (Fig. 1*A*). It has been reported that upon mechanical stimulation (shear stress) the eNOS is responsible for NO production in bone cells (38). As shown in Fig. 1*B*, ultrasound also slightly enhanced eNOS expression in osteoblasts. In addition, exposure of MC3T3-E1 to US for different time intervals also led to a time-dependent increase in NO production (Fig. 1*C*). Exposure to US for 20 min was most efficient to induce NO release in osteoblasts (Fig. 1*C*). The US effect on iNOS expression was much more prominent than that on eNOS expression. We thus examined the signaling pathways involved in the up-regulation of iNOS after US stimulation in the following sections. It has been reported that integrins function as mechanoreceptors that detect mechanical stimuli originating from the extracellular matrix and convert them to chemical signaling pathways that regulate cell functions (6, 11). We then examined whether $\alpha5\beta1$ and $\alpha v\beta3$ integrin act as mechanoreceptors detecting mechanical stimuli from the US stimulation and lead to the increase of iNOS expression in osteoblasts. Pretreatment of osteoblasts for 30 min with monoclonal antibodies (mAbs) against $\alpha5\beta1$ or $\beta1$ integrin but not $\alpha v\beta3$ (Clone H9.2B) or $\beta3$ integrin (Clone 2C9.2G2, HMβ3-1) antagonized the US-induced iNOS expression (Fig. 1, *D* and *E*). The cyclic RGD peptide (cyclo-RGDfV) has been reported to bind $\alpha v\beta3$ at high affinity and block its function effectively at low concentrations (42, 43). Pretreatment of cells with cyclic

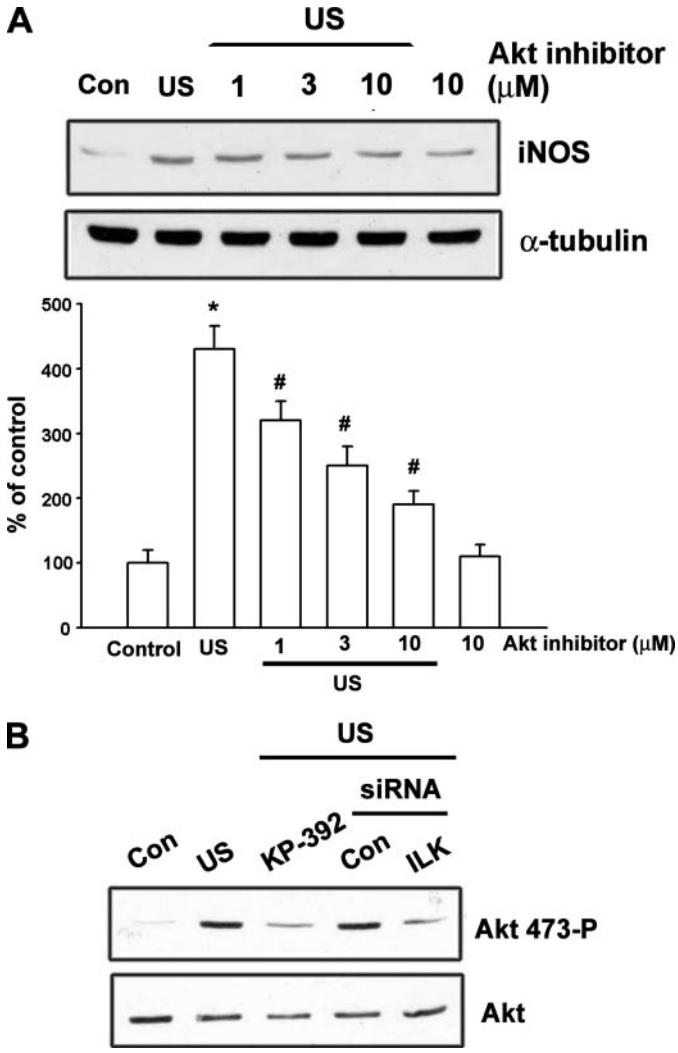

FIGURE 3. **Akt is involved in the US-mediated increase of iNOS expression.** *A*, osteoblasts were pretreated with Akt inhibitor (1–10 μM) for 30 min followed by stimulation with US for 20 min, and the iNOS expression was determined by immunoblotting at 12 h following US. The quantitative data are shown in the *lower panels* ($n = 4$). *B*, cells were pretreated with KP-392 (30 μM) for 30 min or transfected with ILK siRNA for 24 h followed by exposure to US for 20 min, and Akt phosphorylation was then determined 15 min after US stimulation. Typical traces represent three experiments with similar results. Results are expressed as the mean ± S.E. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with US treatment alone.

RGD peptide (30 μM) or cyclic RAD (30 μM, negative control) also did not affect the US-increased iNOS expression (Fig. 1*E*). FAK has been shown to be capable of regulating US-induced increase COX-2 expression in osteoblasts (6). Transfection with FAK(Y397F) mutant for 24 h did not affect the US-induced iNOS expression in osteoblasts (Fig. 1*F*). Taken together, these results indicate that the $\beta1$ integrin but not $\beta3$ integrin or FAK pathway is involved in US-induced iNOS expression.

The Signaling Pathways of ILK, Akt, and mTOR Are Involved in the Potentiating Action of US Stimulation—ILK, a 59-kDa serine/threonine protein kinase that interacts with cytoplasmic domain of both $\beta1$- and $\beta3$-integrins, is activated by cell-extracellular matrix interactions (14). To explore whether ILK is involved in US-induced iNOS expression, ILK inhibitor KP-392 or ILK siRNA were used. As shown in Fig. 2*A*, pretreatment of osteoblasts with KP-392 inhibited US-induced iNOS expres-

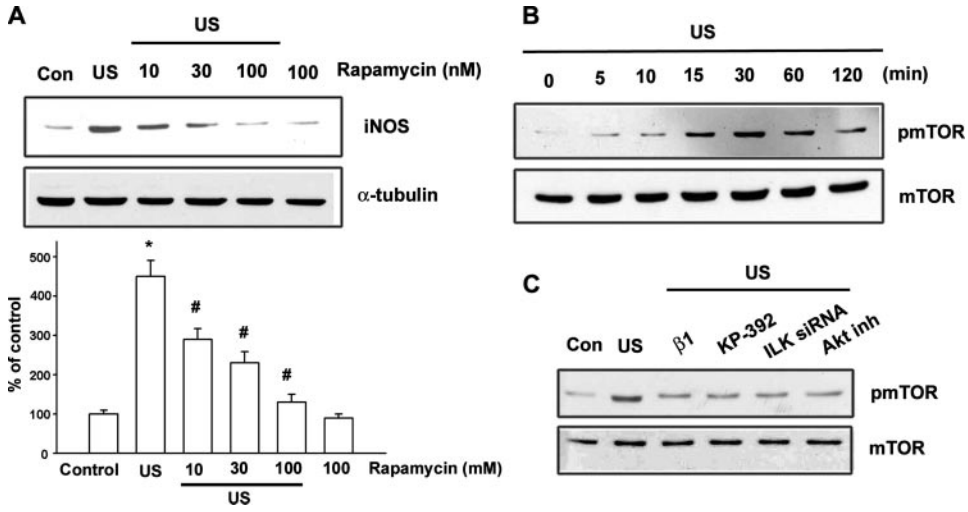

FIGURE 4. **Involvement of mTOR signaling pathway in response to US stimulation in osteoblast.** *A*, osteoblasts were pretreated with mTOR inhibitor of rapamycin (10–100 nM) for 30 min followed by stimulation with US for 20 min, and iNOS protein level was determined by immunoblotting 12 h following US. The quantitative data are shown in the *lower panels* ($n = 3$). *B*, osteoblasts were stimulated with US for 20 min, and then mTOR phosphorylation was determined at various time intervals after US stimulation. Typical traces represent three experiments with similar results. *C*, cells were pretreated with mAb against β1 integrin (20 μg/ml), KP-392 (30 μM), Akt inhibitor (3 μM) for 30 min or transfected with ILK siRNA for 24 h followed by exposure to US for 20 min, and mTOR phosphorylation was then determined 30 min after US stimulation. The protein level of mTOR is shown for comparison. Typical traces represent three experiments with similar results. Results are expressed as the mean ± S.E. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with US treatment alone.

sion in a concentration-dependent manner. Transfection of osteoblasts with ILK siRNA also antagonized the potentiating effect of US (Fig. 2*B*). We thus directly measured the ILK kinase activity in response to US stimulation by the immunoprecipitation of ILK from lysates. Fig. 2*C* shows that US exposure in osteoblasts time-dependently increased ILK kinase activity, assessing the phosphorylation of the recombinant GSK3β on Ser^{9}. It has been reported that ILK is an upstream regulator of the phosphorylation of Akt on Ser^{473} (28), we then examined whether US stimulation also enhances the association of ILK with Akt. Pretreatment of cells for 30 min with Akt inhibitor (1*L*-6-hydroxymethyl-*chiro*-inositol-2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate], 1–10 μM) inhibited the US-induced iNOS expression in a concentration-dependent manner (Fig. 3*A*). US-induced Akt phosphorylation on Ser^{473} markedly decreased if osteoblasts were pretreated for 30 min with KP-392 or transfected for 24 h with ILK siRNA (Fig. 3*B*). To examine whether mTOR activation is involved in the elevation of iNOS expression caused by US stimulation, the mTOR inhibitor rapamycin were used. Pretreatment with rapamycin (10–100 nM) antagonized the US-induced increase of iNOS expression in a concentration-dependent manner (Fig. 4*A*). After exposure to US mTOR, phosphorylation on Ser^{2448} increased at 5 min, reaching maximum between 15 and 120 min (Fig. 4*B*). US-induced mTOR phosphorylation was markedly inhibited by the pretreatment of cells for 30 min with mAb against β1 integrin, KP-392, and Akt inhibitor or transfection with ILK siRNA for 24 h. Taken together, these results indicated that the β1 integrin/ILK/Akt/mTOR pathway is involved in US-induced iNOS expression.

US Promotes HIF-1 Protein Stability and Induces HIF-1 Activation—HIF, a pivotal transcription factor, is a dominant regulator of iNOS gene expression (29). We therefore sought to determine whether HIF was involved in US-induced iNOS expression in the cultured osteoblasts. To this end, MC3T3-E1 cells were exposed to US for 20 min, and the cell lysates were collected at different time intervals. The results from Western blot analysis and reverse transcription-PCR indicated that US significantly increased protein level of HIF-1α time dependently but not at mRNA level (Fig. 5, *A* and *B*). Regulation of HIF-1α seems to occur principally at the protein level by HIF-1α stabilization and activation (30). We therefore attempted to determine HIF-1α protein stability and activity in response to US stimulation in MC3T3-E1 cells. To analyze the effect of US on HIF-1α protein stability, MC3T3-E1 cells were exposed with US for 20 min, after which cycloheximide was added for a further 30–120 min to block ongoing protein synthesis. According to the Western blot analyses conducted thereafter, the half-life of HIF-1α protein seemed to be >100 min for US-treated cells and <50 min for control cells (Fig. 5*C*). These results suggest that US increases HIF-1α protein, possibly by enhancing HIF-1α protein stability.

Nuclear translocation of HIF-1α is necessary for its transcriptional activation of a variety of HIF-1-regulated genes, including iNOS (29). We therefore examined the nuclear translocation of HIF-1α protein in MC3T3-E1 cells after treatment with US by Western blot analysis. As shown in Fig. 5*D*, US stimulation enhanced the accumulation of HIF-1α in the cytosol and the translocation into the nucleus in a time-dependent manner. Thereafter, we performed DNA affinity protein binding assay and ChIP assay to examine the DNA binding activity of HIF-1α in US-treated cells. DNA affinity protein binding assay experiments showed a time-dependent increase in the binding of HIF-1α to the HRE element on the iNOS promoter after US stimulation for 20 min (Fig. 5*E*). The *in vivo* recruitment of HIF-1α to the iNOS promoter was assessed by ChIP assays. *In vivo* binding of HIF-1α to the HRE element of iNOS promoter occurred as early as 10 min and sustained to 120 min after US stimulation (Fig. 5*F*). Based upon these findings, we suggest that US enhances the protein stability and DNA binding activity of HIF-1α.

US-induced HIF-1 Activation and Subsequent iNOS Expression via Integrin Receptor-mediated ILK/Akt and mTOR Pathways—We further explored whether integrin/ILK/Akt and mTOR pathways were involved in the US-induced HIF-1α activation in the cultured osteoblasts. US mediated an increase of HIF-1α expression, and HIF-1α binding to the HRE element by US exposure was inhibited by β1 integrin mAb, KP-392, Akt inhibitor, and rapamycin, as shown by Western blot, DNA affinity protein binding assay, and ChIP assay, respectively (Fig.

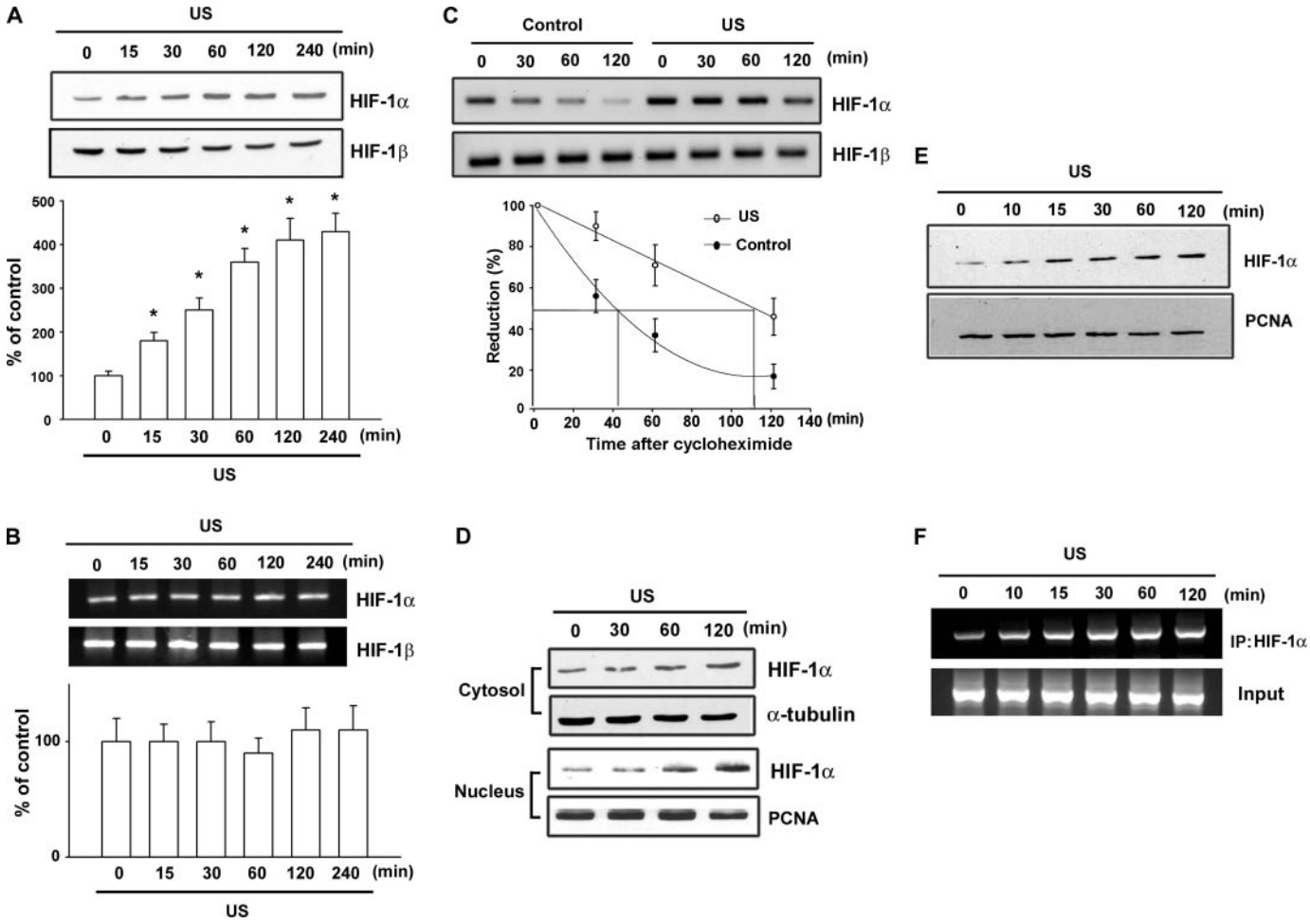

FIGURE 5. **US enhances HIF-1α protein stability and induces HIF-1α activation.** *A*, osteoblasts were exposed to US for 20 min. Protein level of HIF-1α was measured 15, 30, 60, 120, and 240 min after US treatment. The quantitative data are shown in the *lower panels* ($n = 3$). *B*, the cells were stimulated with US for 20 min, and the mRNA for HIF-1α was then analyzed by reverse transcription-PCR. The quantitative data are shown in the *lower panels* ($n = 4$). *C*, osteoblasts were exposed to US for 20 min, after which cycloheximide was added for 30–120 min. Total protein was isolated, and expression of HIF-1α and HIF-1β was analyzed by Western blot assay (*top*). Data were quantified by ImageQuaNT software (Amersham Biosciences) (*bottom*). Results are representative of at least three independent experiments. *, $p \leq 0.05$ as compared with control. *D*, cytosolic and nuclear fractions were prepared from MC3T3-E1 cells exposed to US as described under "Experimental Procedures." Cytosolic and nuclear fractions were subjected to Western bolt analysis by using the indicated antibodies for HIF-1α. α-Tubulin and proliferating cell nuclear antigen (PCNA) acted as the internal loading control for cytosolic and nuclear fractions, respectively. Typical traces represent three experiments with similar results. *E*, osteoblasts were exposed to US for 20 min, and nuclear extracts were prepared and incubated with biotinylated HRE probe at the indicated time intervals after US stimulation. The complexes were precipitated by streptavidin-agarose beads as described under "Experimental Procedures," and HIF-1α in the complexes was detected by Western blot. The equal amount of input nuclear protein was examined by the PCNA protein level. Typical traces represent three experiments with similar results. *F*, cells were exposed to US for 20 min, and nuclear extracts were prepared at indicated time after US stimulation, and ChIP assay was then performed. Chromatin was immunoprecipitated with HIF-1α antibody. One percent of the precipitated chromatin was assayed to verify equal loading (*Input*). Typical traces represent three experiments with similar results. Results are expressed as the mean ± S.E. *, $p \leq 0.05$ as compared with control.

6, *A–C*). Pretreatment of cells with β1 integrin mAb, KP-392, Akt inhibitor, and rapamycin or transfection with ILK siRNA, Akt, and HIF-1α mutant also antagonized the US-induced increase in HRE luciferase activity (Fig. 6, *D* and *E*).

To further study the pathways involved in the action of US-induced iNOS expression, transient transfection was performed using the mouse iNOS promoter-luciferase construct, piNOS-Luc, which contains the mouse iNOS gene between positions −1592 and +171 fused to the luciferase reporter gene. Exposure to US led to a 3.2-fold increase in iNOS promoter activity in osteoblasts. The increase of iNOS activity by US stimulation was antagonized by mAb against β1 integrin (20 μg/ml), KP-392 (30 μM), Akt inhibitor (3 μM), and rapamycin (30 nM) (Fig. 7*A*). In co-transfection experiments, the increase of iNOS promoter activity by US was inhibited by the ILK siRNA or dominant negative mutant of Akt and HIF-1α (Fig. 7*B*). Taken together, these data suggest that the activation of the β1 integrin/ILK/Akt/mTOR/HIF-1α pathway is required for the US-induced increase of iNOS in osteoblasts.

DISCUSSION

Bone cells are equipped with mechanisms to sense diverse physical forces and transduce signals for adjustment to their microenvironment (31). The non-invasive nature of US provides many advantages in practical applications. Although US is clinically used as a treatment for fracture repair, the molecular mechanisms by which US alters cell functions, *e.g.* protein metabolism, are virtually unknown. Our previous data show that US exposure transiently increases the membrane expression of integrins and results in the expression of COX-2 and

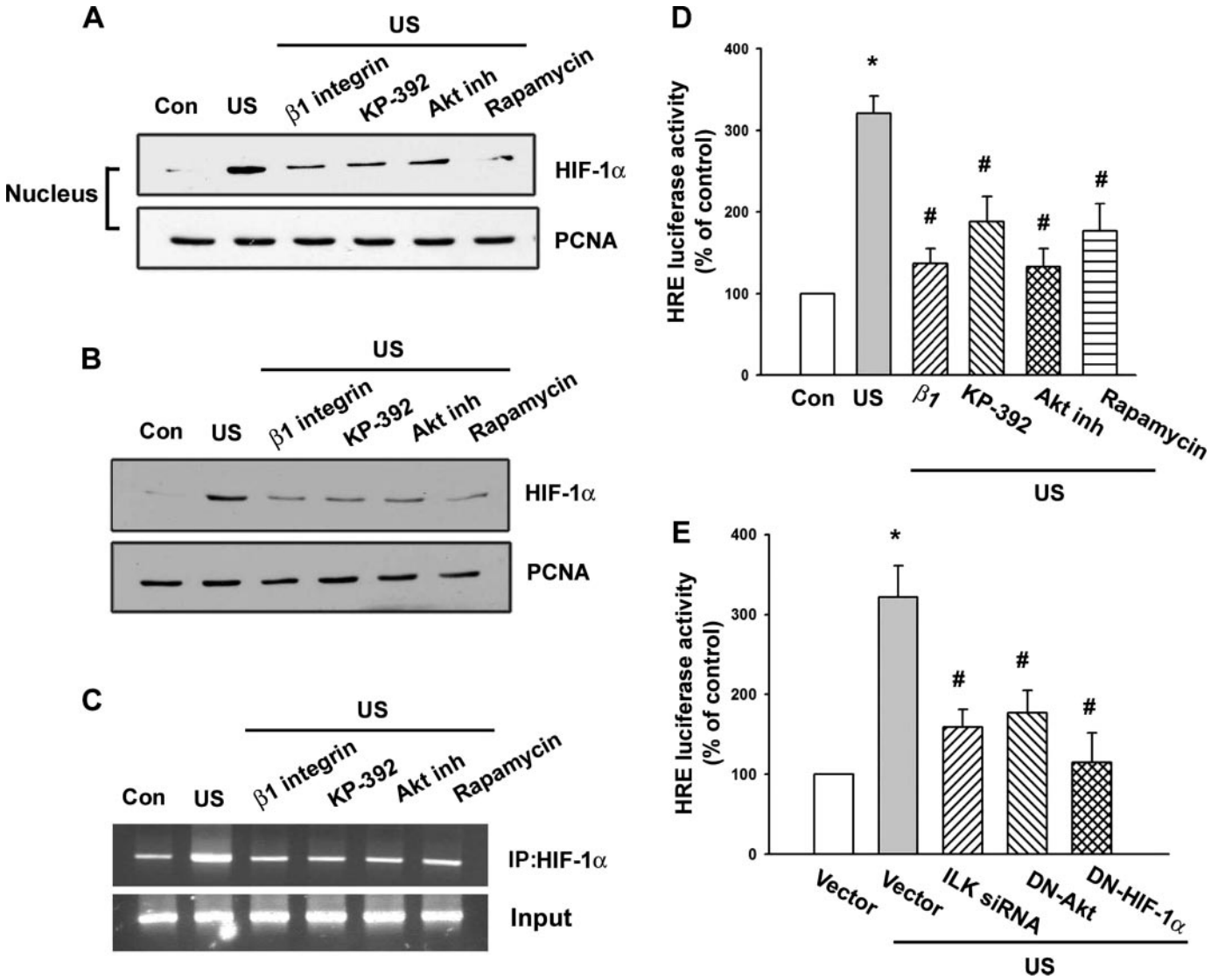

FIGURE 6. **Integrin/ILK/Akt and mTOR pathways are involved in US-induced HIF-1α activation.** *A*, cells were pretreated with mAb against β1 integrin (20 μg/ml), KP-392 (30 μM), Akt inhibitor (3 μM), or rapamycin (30 nM) for 30 min followed by exposure to US for 20 min, and nuclear extracts were prepared at indicated time intervals after US stimulation. The nuclear fractions were subjected to Western blot analysis by using indicated antibodies for HIF-1. Typical traces represent three experiments with similar results. *B*, cells were pretreated with mAb against β1 integrin (20 μg/ml), KP-392 (30 μM), Akt inhibitor (3 μM), or rapamycin (30 nM) for 30 min followed by exposure to US for 20 min, and nuclear extracts were prepared and incubated with biotinylated HRE probe at indicated time interval after US stimulation. The complexes were precipitated by streptavidin-agarose beads as described under "Experimental Procedures," and HIF-1α in the complexes was detected by Western blot. The equal amount of input nuclear protein was examined by the PCNA protein level. Typical traces represent three experiments with similar results. *C*, cells were pretreated with mAb against β1 integrin (20 μg/ml), KP-392 (30 μM), Akt inhibitor (3 μM), or rapamycin (30 nM) for 30 min followed by exposure to US for 20 min, and ChIP assay was then performed. Chromatin was immunoprecipitated with HIF-1α antibody. One percent of the precipitated chromatin was assayed to verify equal loading (*Input*). Typical traces represent three experiments with similar results. Osteoblasts were pretreated with mAb against β1 integrin (20 μg/ml), KP-392 (30 μM), Akt inhibitor (3 μM), or rapamycin (30 nM) for 30 min (*D*) or transfected with ILK siRNA or DN mutant of Akt or HIF-1α (*E*) for 24 h and then treated for 20 min with US. Luciferase activity was measured 24 h after US stimulation, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with US treatment alone.

synthesis of prostaglandin E_2 (6). In this study, we further identify iNOS as a target protein for US signaling pathway in cultured osteoblasts. Furthermore, the present study suggests a role of β1 integrin in the transduction of the acoustic pressure that leads to the expression of iNOS after US exposure. Klein-Nulend *et al.* (5), has also reported that US increased NO production via the iNOS-dependent pathway. On the other hand, mechanical loading has been reported to induce NO production through eNOS signal (38). Our data also show that ultrasound stimulation slightly increased eNOS expression. Therefore, the NO production by US stimulation may involve both iNOS and eNOS signaling pathways. We have focused on the signaling pathways involved in up-regulation of iNOS. Whether the similar signaling pathways are involved in US-induced eNOS expression needs further investigation.

Ultrasound stimuli are transferred to adherent cells through their adhesive contacts with surrounding extracellular matrix. Integrins act as a link between extracellular matrix, cytoskeletal proteins, and actin filaments (32). We previously found that US treatment transiently increased the cell-surface expression of α2, α5, β1, and β3 but not α3 and α4 integrins (6). Moreover, treatment with anti-α5β1 or -αvβ3 mAbs antagonized the

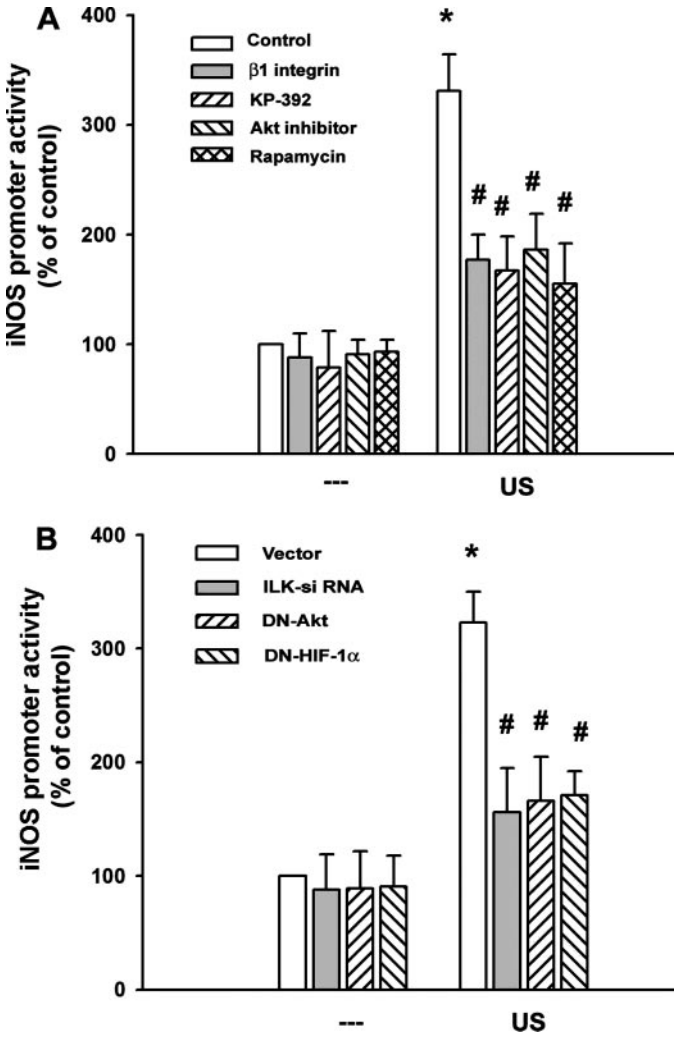

FIGURE 7. **Involvement of integrin/ILK/Akt/mTOR/HIF-1α in the increase of iNOS promoter activity by US stimulation.** *A*, the iNOS promoter activity was evaluated by transfection with the piNOS-Luc luciferase expression vector. Osteoblasts were pretreated with mAb against β1 integrin (20 μg/ml), KP-392 (30 μM), Akt inhibitor (3 μM), or rapamycin (30 nM) for 30 min before stimulation with US. *B*, cells were co-transfected with piNOS-Luc and the ILK siRNA or DN mutant of Akt or HIF-1α and then treated for 20 min with US. Luciferase activity was measured 24 h after US stimulation, and the results were normalized to the β-galactosidase activity and expressed as the mean $\pm$ S.E. for three independent experiments performed in triplicate. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with US treatment alone.

potentiating action of US stimulation on COX-2 expression (6). However, the results of our current study showed that $\alpha5\beta1$ or $\beta1$ integrin mAbs but not $\alpha v\beta3$ or $\beta3$ integrin mAbs inhibited the US-induced increase in iNOS expression, indicating that $\beta1$ integrin is very important for mediating the action of US in osteoblasts. It has been found that cyclical pressure-induced strain results in rapid tyrosine phosphoregulation of FAK, paxillin, and β-catenin in human articular chondrocytes (33). Our previous data also demonstrate that US stimulation increases phosphorylation of tyrosine 397 of FAK (6). Here we found that the FAK(Y397F) mutant did not antagonize the US-mediated potentiation of iNOS expression, suggesting that FAK activation is not an obligatory event in US-induced iNOS expression in these cells. Our data implied that the $\beta1$ integrin but not $\beta3$ integrin or FAK signaling pathways is involved in US-induced iNOS expression.

Cell-extracellular matrix interaction can activate ILK, which interacts with the cytoplasmic domain of both $\beta1$ and $\beta3$ integrins (14). ILK has been reported to regulate integrin-mediated cell adhesion and invasion, E-cadherin expression, and extracellular matrix assembly (34). Tan *et al.* (39) have shown that overexpression or activation of ILK increased the expression of iNOS in macrophages. Because FAK mutant did not affect US-induced iNOS expression, we then examined whether the ILK is involved in US-induced increase of iNOS expression. The current study showed that US stimulation increased kinase activity of ILK. Treatment with the ILK inhibitor of KP-392 inhibited US-induced iNOS expression. Furthermore, the ILK siRNA also antagonized the US-mediated potentiation of iNOS expression. Therefore, ILK activation is involved in US-induced iNOS expression in the cultured osteoblasts. ILK possibly regulated the cell function by promoting the phosphorylation of Akt on Ser^{473} and its downstream pathways of mTOR (35). Our results demonstrated that pretreatment of osteoblasts with Akt or mTOR inhibitors antagonized the increase of iNOS expression by US stimulation. This was further confirmed by the result that the dominant negative mutant of Akt inhibited the enhancement of iNOS promoter activity by US stimulation. We previously found that phosphorylation of Akt on Ser^{473} increased in a time-dependent manner in response to US stimulation (6). Here we also found that the cytoplasmic serine kinase mTOR was activated by US stimulation in osteoblastic cells. These effects were inhibited by mAb against $\beta1$ integrin, KP-392 or Akt inhibitor, or ILK siRNA, indicating the involvement of integrin/ILK/Akt-dependent mTOR activation in US-mediated induction of iNOS. Activation of the ILK/Akt/mTOR-dependent pathway has also been reported to regulate vascular epidermal growth factor in tumor angiogenesis (22). Matrix-derived mechanical forces sensed by $\beta1$ integrin are capable of modulating the ILK/Akt-dependent mechanism, which regulates fibroblast viability (37). In addition, regulation of tumor growth by ILK inhibitor (QLT0254) is also related to the ILK/Akt/mTOR signal cascade (36). Taken together, our results provide evidence that US up-regulates iNOS in the cultured osteoblasts via the integrin/ILK/Akt/mTOR signaling pathway.

HIF-1α has been reported to activate iNOS expression by binding to the HRE site within the iNOS promoter in response to hypoxia in microglia cells (29). Our previous studies found that exposure of osteoblasts to US result in IκBα phosphorylation and IκBα degradation in the cytosol and the translocation of p65 from cytosol to nucleus. US stimulation also increased NF-κB luciferase activity in osteoblasts (6). However, the effect of US stimulation on HIF-1α activity in osteoblast is mostly unknown. Using transient transfection with HRE-luciferase as an indicator of HIF-1α activity, we found that US increased HIF-1α activity in cultured osteoblasts. Transfection with HIF-1α mutant also inhibited US-induced increases in iNOS luciferase activity, indicating that HIF-1α activation contributes to US-induced iNOS induction in osteoblasts. Furthermore, US stimulation increased the binding of HIF-1α to the HRE element on iNOS promoter, as shown by DNA affinity protein binding assay and ChIP assay. Binding of HIF-1α to the HRE element was attenuated by mAb against $\beta1$ integrin, ILK, Akt, and rapamycin inhibitor. These results indicate that US

might act through the integrin, ILK, Akt, mTOR, and HIF-1α pathway to induce iNOS activation in osteoblasts.

In conclusion, the signaling pathway involved in US-induced iNOS expression in osteoblasts has been explored. US stimulated up-regulation iNOS levels through the activation of HIF-1α by the integrin-dependent ILK/Akt and mTOR signaling pathways.

Acknowledgments—We thank Dr. E. A. Ratovitski for providing the piNOS-luciferase construct, Dr. M. L. Kuo for providing the pHRE-luciferase construct, Dr. J. A. Girault for providing an FAK mutant, and Dr. R. H. Chen for providing an Akt dominant negative mutant. We also thank Smith & Nephew Co. (Memphis, TN) for providing 6-well ultrasound devices.

REFERENCES

1. Duarte, L. R. (1983) *Arch. Orthop. Trauma Surg.* **101,** 153–159
2. Wang, S. J., Lewallen, D. G., Bolander, M. E., Chao, E. Y., Ilstrup, D. M., and Greenleaf, J. F. (1994) *J. Orthop. Res.* **12,** 40–47
3. Heckman, J. D., Ryaby, J. P., McCabe, J., Freym, J. J., and Kilcoyne, R. F. (1994) *J. Bone Joint Surg.* **76,** 26–34
4. Cook, S. D., Ryaby, J. P., McCabe, J., Frey, J. J., Heckman, J. D., and Kristiansen, T. K. (1997) *Clin. Orthop. Relat. Res.* **337,** 198–207
5. Reher, P., Harris, M., Whiteman, M., Hai, H. K., and Meghji, S. (2002) *Bone* **31,** 236–241
6. Tang, C. H., Yang, R. S., Huang, T. H., Lu, D. Y., Chuang, W. J., Huang, T. F., and Fu, W. M. (2006) *Mol. Pharmacol.* **69,** 2047–2057
7. Yang, R. S., Lin, W. L., Chen, Y. Z., Tang, C. H., Huang, T. H., Lu, B. Y., and Fu, W. M. (2005) *Bone* **36,** 276–283
8. Ruoslahti, E. (1996) *Tumor Biol.* **17,** 117–124
9. Giancotti, F. G., and Ruoslahti, E. (1999) *Science* **285,** 1028–1032
10. Dimmler, S., Assmus, B., Hermann, C., Haendeler, J., and Zeiher, A. M. (1998) *Circ. Res.* **83,** 334–341
11. Tian, B., Lessan, K., Kahm, J., Kleidon, J., and Henke, C. (2002) *J. Biol. Chem.* **277,** 24667–24675
12. Plopper, G. E., McNamee, H. P., Dike, L. E., Bojanowski, K., and Ingber, D. E. (1995) *Mol. Biol. Cell* **6,** 1349–1365
13. Chicurel, M. E., Chen, C. S., and Ingber, D. E. (1998) *Curr. Opin. Cell Biol.* **10,** 232–239
14. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., and Filmus, J. (1996) *Nature* **379,** 91–96
15. Wu, C., and Dedhar, S. (2001) *J. Cell Biol.* **155,** 505–510
16. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5510–5514
17. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., and Cockman, M. E. (1999) *Nature* **399,** 271–275
18. Salceda, S., and Caro, J. (1997) *J. Biol. Chem.* **272,** 22642–22647
19. Sharp, F. R., and Bernaudin, M. (2004) *Nat. Rev. Neurosci.* **5,** 437–448
20. Warden, S. J., Favaloro, J. M., Bennell, K. L., McMeeken, J. M., and Zajac, J. D. (2001) *Biochem. Biophys. Res. Commun.* **286,** 443–450
21. van't Hof, R. J., and Ralston, S. H. (2001) *Immunology* **103,** 255–261
22. Tan, C., Cruet-Hennequart, S., Troussard, A., Fazli, L., Costello, P., Sutton, K., Wheeler, J., Gleave, M., Sanghera, J., and Dedhar, S. (2004) *Cancer Cell* **5,** 79–90
23. Tang, C. H., Yang, R. S., and Fu, W. M. (2005) *J. Biol. Chem.* **280,** 22907–22916
24. Melillo, G., Taylor, L. S., Brooks, A., Musso, T., Cox, G. W., and Varesio, L. (1997) *J. Biol. Chem.* **272,** 12236–12243
25. Huang, W. C., and Chen, C. C. (2005) *Mol. Cell Biol.* **25,** 6592–6602
26. Deng, W. G., Tang, S. T., Tseng, H. P., and Wu, K. K. (2006) *Blood* **108,** 518–524
27. Saito, M., Soshi, S., Tanaka, T., and Fujii, K. (2004) *Bone* **35,** 644–655
28. Troussard, A. A., Mawji, N. M., Ong, C., Mui, A., St. Arnaud, R., and Dedhar, S. (2003) *J. Biol. Chem.* **278,** 22374–22378
29. Lu, D. Y., Liou, H. C., Tang, C. H., and Fu, W. M. (2006) *Biochem. Pharmacol.* **72,** 992–1000
30. Jiang, B. H., Rue, E., Wang, G. L., Roe, R., and Semenza, G. L. (1996) *J. Biol. Chem.* **271,** 17771–17778
31. Rubin, C. T., Gross, T. S., McLeod, K. J., and Bain, S. D. (1995) *J. Bone Miner. Res.* **10,** 488–495
32. Hynes, R. O. (1992) *Cell* **69,** 11–25
33. Lee, H. S., Millward-Sadler, S. J., Wright, M. O., Nuki, G., and Salter, D. M. (2000) *J. Bone Miner. Res.* **15,** 1501–1509
34. Filipenko, N. R., Attwell, S., Roskelley, C., and Dedhar, S. (2005) *Oncogene* **24,** 5837–5849
35. Persad, S., Attwell, S., and Gray, V. (2001) *J. Biol. Chem.* **276,** 27462–27469
36. Yau, C. Y., Wheeler, J. J., Sutton, K. L., and Hedley, D. W. (2005) *Cancer Res.* **65,** 1497–1504
37. Nho, R. S., Xia, H., Kahm, J., Kleidon, J., Diebold, D., and Henke, C. A. (2005) *J. Biol. Chem.* **280,** 26630–26639
38. Klein-Nulend, J., Helfrich, M. H., Sterck, J. G., MacPherson, H., Joldersma, M., Ralston, S. H., Semeins, C. M., and Burger, E. H. (1998) *Biochem. Biophys. Res. Commun.* **250,** 108–114
39. Tan, C., Mui, A., and Dedhar, S. (2002) *J. Biol. Chem.* **277,** 3109–3116
40. Schultz, J. F., and Armant, D. R. (1995) *J. Biol. Chem.* **270,** 11522–11531
41. Dechantsreiter, M. A., Planker, E., Matha, B., Lohof, E., Holzemann, G., Jonczyk, A., Goodman, S. K., and Kessler, H. (1999) *J. Med. Chem.* **42,** 3033–3040
42. Pfaff, M., Tangemann, K., Muller, B., Gurrath, M., Muller, G., Kessler, H., Timpl, R., and Engel, J. (1994) *J. Biol. Chem.* **269,** 20233–20238
43. Brooks, P., Stromblad, S., Sanders, L., Von Schalscha, T., Aimes, R., Stetler-Stevenson, W., Ouigley, J., and Cheresh, D. A. (1996) *Cell* **85,** 683–693

Mechanisms of Signal Transduction:

Ultrasound Induces Hypoxia-inducible Factor-1 Activation and Inducible Nitric-oxide Synthase Expression through the Integrin/Integrin-linked Kinase/Akt/Mammalian Target of Rapamycin Pathway in Osteoblasts

Chih-Hsin Tang, Dah-Yuu Lu, Tzu-Wei Tan,
Wen-Mei Fu and Rong-Sen Yang

J. Biol. Chem. 2007, 282:25406-25415.
doi: 10.1074/jbc.M701001200 originally published online June 21, 2007