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Ultrasound stimulates cyclooxygenase-2 expression and increases bone formation through integrin, FAK, phosphatidylinositol 3-kinase and Akt pathway in osteoblasts

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Running title: Ultrasound stimulates COX-2 and bone formation.

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Abbreviations: COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; DN, dominant negative; ERK, extracellular signal-regulated kinases; FAK, focal adhesion kinase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; NO, nitric oxide; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinase; Rn, rhodostomin US, ultrasound; PD98059, 2'-amino-3'-methoxyflavone; PDTC, pyrrolidine dithiocarbamate.

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Abstract

It has been shown that ultrasound (US) stimulation accelerates fracture healing in the animal models and in clinical studies. Here we found that US stimulation transiently increased the surface expression of α2, α5, β1 and β3 integrins in cultured osteoblasts, as shown by flow cytometric analysis and immuofluorescence staining. US stimulation increased prostaglandin E_2 formation as well as the protein and mRNA levels of cyclooxygenase-2 (COX-2). At the mechanistic level, anti-integrin α5β1 and αvβ3 antibodies or rhodostomin, a snake venom disintegrin, attenuated the US-induced COX-2 expression. Phosphatidylinositol 3-kinase (PI3K) inhibitors, LY294002 and wortmannin also inhibited the potentiating action of US. US stimulation increased the phosphorylation of focal adhesion kinase (FAK), extracellular signal-regulated kinases (ERK), p85 subunit of PI3K and serine 473 of Akt. COX-2 promoter activity was enhanced by US stimulation in cells transfected with pCOX2-Luc. Cotransfection with dominant negative mutant of FAK(Y397F), p85(Δp85) , Akt(K179A) or ERK2(K52R) inhibited the potentiating action of US on COX-2 promoter activity. Expression of mineralized nodule was lower in dominant negative mutants of FAK, p85 and Akt-transfected clones than in vector-transfected control cells. Taken together, our results provide evidence that US stimulation increases COX-2 expression and promotes bone formation in osteoblasts via the integrin/FAK/PI3K/Akt and ERK signaling pathway.

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Introduction

Fracture healing is a complex physiologic process that involves the coordinated participation of several cell types. Among all the means to influence fracture healing, ultrasound (US) 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 (Duarte, 1983; Wang et al., 1994) and clinical studies (Heckman et al., 1994; Cook et al., 1997).

Bone is a dynamic tissue that remodels in response to mechanical loads from the external environment (Rubin and Lanyon, 1985; Turner et al., 1994). While the augmentation of fracture healing by US is well documented, the underlying mechanism of the mechanotransduction pathway involved in cellular responses to US is largely unknown. It has been demonstrated that exposure to low-intensity US pulses increases cyclooxygenase-2 (COX-2) mRNA expression and release of prostaglandin E_2 (PGE_2), which are required for mechanically induced bone formation (Reher et al., 2002). However, the mechanisms involved in osteoblasts to detect US stress and transduce the signal across the membrane for activating signaling pathways in bone metabolism, such as induction of COX-2 and release of PGE_2, remain poorly understood.

Integrins are cell surface adhesion receptors that regulate cell viability in response to cues derived from the extracellular matrix (Ruoslahti, 1996; Giancotti and Ruoslahti, 1999). 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 (Dimmler et al., 1998; Tian et al., 2002). 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 (Plopper et al., 1995; Chicurel et al., 1998). Focal adhesion kinase (FAK), a

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potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling (Crouch et al., 1996; Hadden and Henke, 2000). However, the downstream signaling pathways that mediate integrin-FAK signaling are diverse, and the factors determining which pathway is utilized remain obscure. Ligation and clustering of integrins activate FAK by autophosphorylation of tyrosine 397, creating a potential binding site for the SH2 domains of the p85 subunit of PI3K (Schlaepfer and Hunter, 1998; Eliceiri et al., 2002). Phosphorylation of the p85 subunit of PI3K by FAK may activate the p110 catalytic subunit of PI3K and the PI3K/Akt signaling pathway.

Intracellular signals that promote osteoblast differentiation, including those mediated by bioactive radicals such us nitric oxide (NO), PGE, and calcium, may occur in response to cellular homeostatic disturbance induced by US (Warden et al., 2001; Reher et al., 2002). It has been reported that US exposure increased NO and PGE_2 release via up-regulation of iNOS and COX-2 in osteoblasts (Reher et al., 2002). PGE_2 may enhance the maturation of osteoblast and decrease the formation of osteoclast (Take et al., 2005). However, the signaling pathway for US stimulation on COX-2 expression and bone formation are mostly unknown. Here we found that US stimulation increased expression of integrin in cell membrane. In addition, long-term US stimulation also enhanced osteoblastic differentiation. Furthermore, integrin, FAK, ERK, PI3K and Akt-dependent pathways may be involved in the increase of COX-2 expression and bone formation by US stimulation.

Materials and Methods

Materials:

Rabbit polyclonal antibody for phosphor-FAK (Tyr397) was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibody for COX-2 was purchased from Cayman Chemical (Ann Brbor, MI). Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for COX-1, FAK, p85, p65, IκBα, phosphor-IκBα, ERK 2, phosphor-ERK

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1/2, α-tubulin, phosphotyrosine residues (PY20) and phosphor-Akt (Ser473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for α2, α3, α4, α5, β1, β3 and α5β1 integrin were purchased from Chemicon (Temecula, CA). Mouse monoclonal antibody for αvβ3 was purchased from DAKO (Denmark). Rhodostomin was purified from venom of *Calloselasma rhodostoma* (Huang et al., 1990). The COX-2 promoter construct (pCOX2-Luc) was a gift from Dr. H.R. Herschman (UCLA, CA). The phosphorylation site mutant of FAK(Y397F) was a gift from Dr. J.A. Girault (Institut du Fer à Moulin, France). The p85 (Δp85; deletion of 35 amino acids from residues 479-513 of p85) and Akt (Akt K179A) dominant negative mutant were gifts from Dr. R.H. Chen (Institute of Molecular Medicine, National Taiwan University, Taiwan). The ERK2 (K52R) dominant negative mutant was provided by Dr. M. Cobb (South-Western Medical Center, Dallas, TX). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). The NF-κB-Luc expression plasmid was purchased from Stratagene (La Jolla, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

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-MEM (Gibco, Grand Island, NY) which was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (pH 7.6). The cell medium was changed twice a week.

Primary osteoblastic cells were prepared by the method as previously described (Tang et al., 2003). The calvaria of fetal rats were dissected from fetal rats, divided into small pieces and then treated with 0.1% type I collagenase solution for 10 min at 37°C. The next two 20 min sequential collagenase digestions were then pooled and filtered through 70 μm nylon filters (Falcon, BD BioSciences, San Jose, CA). The cells were grown on the plastic cell culture dishes in 95 % air-5 % CO_2 with α-MEM

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(Gibco, Grand Island, NY) which was supplemented with 20 mM HEPES and 10 % heat-inactivated FBS, 2 mM-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) (pH adjusted to 7.6). The characteristics of osteoblasts were confirmed by morphology and the expression of alkaline phosphatase.

Ultrasound treatment

Cells (3×10^5 cells/well, six-well plates) were cultured for 24 hr and subjected to US treatment. A UV-sterilized transducer (Exogene 2000; Smith & Nephew Inc., Memphis, TN, USA) that generated 1.5 MHz US in a pulsed-wave mode (200 μs burst width with repetitive frequency of 1 kHz at the intensity of 30 mW/cm^2) was immersed vertically into each culture well and placed to just contact the surface of the medium. The distance between the transducer and the cells was approximately 5-6 mm. Exposure time was 20 min/day for all cultures. Control samples were prepared in the same manner without US exposure. Cells were harvested at 1, 3, 6, 12 and 24 hr after US stimulation.

Flow cytometric analysis

Primary osteoblasts or MC3T3-E1 cells were plated in six-well dishes. The cells were then washed with phosphate-buffered saline (PBS) and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsing in PBS, the cells were incubated with rabbit anti-rat antibodies against α2, α3, α4, α5, β1, or β3 integrin (1:100, Chemicon, Temecula, CA) for 1 hr at 4°C. Cells were then washed again and incubated with FITC-conjugated goat anti-rabbit secondary IgG (1:150, Leinco Tec. Inc, St. Louis, MO) for 45 min and analyzed by flow cytometry using FACS Calibur (CellQuest software, BD Biosciences).

Immunocytochemistry

Osteoblasts were grown on glass coverslips. Cultures were rinsed once with PBS, and fixed for 30 sec at room temperature in acetone. Cells were then rinsed

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three times with PBS. After blocking with 4% BSA for 15 min, cells were incubated with rabbit anti-rat β1 or β3 integrin (1:100) for 1 hr at room temperature. Cells were then washed again and labeled with FITC-conjugated goat anti-rabbit IgG for 1 hr. Finally, cells were washed, mounted and examined with a Leica TCS SP2 Spectral Confocal System.

Immunoprecipitation and Western Blot Analysis

The cellular lysates were prepared as described previously (Tang et al., 2005). Equal amounts of protein were incubated with specific antibody immobilized onto protein A/G-Sepharose for 12 hr at 4°C with gentle rotation. Beads were washed extensively with lysis buffer, boiled, and microcentrifuged. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 hr at room temperature and then probed with rabbit anti-rat antibodies against COX-1, COX-2, pFAK, p85 or pAkt (1:1000) for 1 hr at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 hr at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). For normalization purposes, the same blot was also probed with mouse anti-rat α-tubulin antibody (1:1000). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Quantitative Real Time 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, quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). 100 ng of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers

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and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems, CA). qPCR assays were carried out in triplicate on an ABI Prism 7900 sequence detection system. The cycling conditions were 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as C_T).

PGE_2 assay

Primary osteoblast or MC3T3-E1 cells cultured in 6-well plate were stimulated with US. PGE_2 production was measured by commercial ELISA kit (Cayman Chemical Company) according to manufacturer's instruction.

Transfection and reporter gene assay

Osteoblasts were cotransfected with 1 μg COX-2 promoter plasmid and 1 μg β-galactosidase expression vector. Osteoblasts were grown to 70% confluent in 6-well plates and were transfected on the following day by Lipofectamin 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 hr incubation, transfection was complete, and cells were incubated with the indicated agents. The media were removed 24 hr after US stimulation, and cells were washed once with cold PBS. To prepare lysates, 100 μl of reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 30 sec. 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

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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 FAK(Y397F), p85, Akt or ERK2 mutant or the empty vector (1.0 μg).

Selection of stably transfected clone

Purified plasmid DNA (3 μg) was transfected into MC3T3-E1 cells with LF2000 transfection reagent. Twenty-four hr after transfection, stable transfectants were selected in Gentamicin (G418; Life Technologies) at a concentration of 600 μg/mL. Thereafter, the selection medium was replaced every 3 days. After 2 weeks of selection in G418, clones of resistant cells were isolated and allowed to proliferate in medium containing G418 (100 μg/mL). Integration of transfected plasmid DNA was confirmed by reverse transcription-polymerase chain reaction and Western blot analysis.

Measurement of mineralized nodule formation

Osteoblasts were cultured in α-MEM containing 50 μg/ml vitamin C and 10 mM β-glycerophosphate, and the medium was changed every 3 days. Ultrasound stimulation was applied every day (20 min/day) and bone nodule formation was determined on Day-10 using alizarin red-S staining. Briefly, the ethanol-fixed cells and matrix was stained for 1 hr with 40 mM alizarin red-S. (pH 4.2) and extensively rinsed with water. After photography, the bound staining was eluted with 10% (wt/vol) cetylpyridinium chloride, and alizarin red-S in samples was quantified by measuring absorbance at 550 nm and calculated according to a standard curve. One mol of alizarin red-S selectively binds about 2 mol of calcium (Yang et al., 2005).

Statistics

The values given are means ± S.E.M. 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.

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Results

Ultrasound stimulation increased the expression of integrins in osteoblasts

At the cell surface, integrins exert as signaling molecules and are intimately involved in constructing fibrillar matrix networks from soluble secreted matrix proteins. We used flow cytometry to investigate the effect of US on the expression of integrins in cell membrane. As shown in Fig. 1A, 20 min after US stimulation in MC3T3-E1 cells the fluorescence intensity of α5, β1, and β3 integrins increased, starting and reaching peak between 3 and 6 hr. The fluorescence intensity then gradually declined to resting level. We then used primary cultured osteoblasts to examine the effect of US on the integrin expression in cell membrane. Similar change of the integrin expression was obtained in primary osteoblasts, including α5, β1, and β3 integrins (Fig. 1B). Therefore, US stimulation may transiently increase the integrin expression on cell surface of osteoblasts. We then used immunocytochemistry to visualize the localization of integrins. The β1 or β3 staining in control cells shows a punctate pattern (Fig. 1C). However, the clustering of β1 or β3 integrin was markedly enhanced 6 hr after US stimulation (20 min duration) and the staining of β integrin shows fibrillar pattern (Fig. 1C). In addition, US stimulation also increased the integrin expression of α2 but not α3 and α4 (Fig. 1D), suggesting that US does not up-regulate the expression of all kinds of integrin in osteoblasts. As osteoblasts were cultured on 6-well plates pre-coated with fibronectin, vitronectin and collagen (10 μg/ml, 500 μl for each) for 24 hr, the increase of α5, β1, and β3 integrin expression on cell surface of osteoblasts is similar 6 hr after US stimulation (Fig. 1E). These results indicate that increase of integrin expression on the cell membrane of osteoblasts by US is not affected by pre-coated extracellular matrix.

Effect of ultrasound stimulation on COX-2 expression in osteoblasts

It has been demonstrated that pulse application of low-intensity US increased PGE_2 release, which is important for mechanically induced bone formation. We then

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investigated the effect of US stimulation on the COX-2 expression in osteoblasts. MC3T3-E1 or primary osteoblast cells were exposed to US for 20 min and the cell lysate were collected at different time intervals. The results from Western blot and qPCR indicated that US significantly increased both protein and mRNA levels of COX-2 time-dependently (Fig. 2A, B). The increase of mRNA occurs earlier than that of protein levels. On the other hand, US stimulation did not affect the protein expression of COX-1 in osteoblasts. Furthermore, exposure of MC3T3-E1 or primary osteoblasts to US for 20 min also led to a time-dependent increase in PGE_2 production (Fig. 2C).

The signaling pathways of FAK, PI3K and Akt are involved in the potentiating action of US stimulation

The results shown above demonstrate that US stimulation increases the expression of integrins as well as COX-2. We then examined whether integrin acts as mechanoreceptors detecting mechanical stimuli from the US stimulation and leads to the increase of COX-2 expression in osteoblasts. Pretreatment of osteoblasts for 30 min with monoclonal antibodies (mAb) against α5β1 or αvβ3 (20 μg/ml), Arg-Gly-Asp (RGD)-dependent disintegrin rhodostomin (0.28 μM), or transfection with the FAK dominant negative mutant (Y397F) for 24 hr antagonized the US-induced COX-2 expression (Fig. 3A). FAK has been shown to be capable of regulating integrin-mediated signaling (Hadden and Henke, 2000). Phosphorylation of tyrosine 397 of FAK has been used as a marker of FAK activity. As shown in Fig. 3B, FAK phosphorylation increased in a time-dependent manner in response to 20 min US stimulation, reaching maximum between 10-30 min (Fig. 3B). To determine the relationship of signaling pathway among α5β1, αvβ3 integrins and FAK in response to US stimulation, cells were pretreated for 30 min with mAb against α5β1, αvβ3 (20 μg/ml) or rhodostomin (0.28 μM) or transfection with FAK(Y397F) mutant for 24 hr and the results showed that US-induced FAK phosphorylation was markedly inhibited (Fig. 3C). Phosphorylation of tyrosine 397 of FAK may provide a binding site for the

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Src homology 2 domain of the p85 subunit of PI3K (Chan et al., 1999). Since enforced activation of α5β1 or αvβ3 integrin by US stimulation potentiates FAK phosphorylation, we then examined whether US stimulation also enhances the association of FAK with PI3K. Exposure of osteoblasts to US for 20 min led to a significant increase of phosphorylation of p85 subunit of PI3K, as assessed by measurement of phosphotyrosine from immunoprecipitated lysates using p85 (Fig. 4A). Pretreatment of osteoblasts for 30 min with mAb against α5β1, αvβ3 (20 μg/ml), rhodostomin (0.28 μM) or transfection with FAK(Y397F) mutant markedly attenuated the US-induced p85 phosphorylation (Fig. 4B). To explore whether PI3K is involved in US-induced COX-2 expression, PI3K inhibitors LY294002 and wortmannin were used. As shown in Fig. 5A, pretreatment of osteoblasts with LY294002 and wortmannin inhibited US-induced COX-2 expression in a concentration-dependent manner. Transfection of osteoblasts with p85(Δp85) or Akt (K179A) mutant also antagonized the potentiating effect of US. We thus directly measured the Akt phosphorylation in response to US stimulation. Fig. 5B shows that US exposure in osteoblasts increased Akt phosphorylation (serine 473) in a time-dependent manner. Maximal activation was detected 15 min following US stimulation (20 min duration). Furthermore, the US-induced increase in Akt phosphorylation was markedly inhibited by the pretreatment of cells for 30 min with mAb against α5β1, αvβ3 (20 μg/ml) or rhodostomin (0.28 μM) or transfection with FAK(Y397F) or p85(Δp85) mutant for 24 hr. Taken together, these results indicate that integrin/FAK/PI3K/Akt pathway is involved in US-induced COX-2 expression.

Involvement of ERK and NF-κB in US-induced COX-2 expression

In order to examine whether extracellular signal-regulated kinases (ERK) and NF-κB activation are involved in the elevation of COX-2 expression caused by US stimulation, the MEK inhibitor PD 98059 and the specific NF-κB inhibitor PDTC were used. Pretreatment of cells for 30 min with PD 98059 (10, 30 μM) or PDTC (10, 30 μM) inhibited the US-induced increase in COX-2 expression in a

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concentration-dependent manner (Fig. 6A). ERK phosphorylation increased after 20 min US stimulation, starting at 5 min, reaching maximum between 15-30 min and then gradually decreased (Fig. 6B). US-induced ERK phosphorylation was markedly inhibited if osteoblasts were pretreated for 30 min with mAb against α5β1, αvβ3 (20 μg/ml) or transfection with FAK(Y397F). However, US-induced ERK phosphorylation was not affected when osteoblasts were transfected with p85(Δp85) or Akt(K179A) mutant (Fig. 6C). These results indicate that α5β1 or αvβ3 integrins and FAK but not PI3K and Akt are upstream regulators of ERK in US-induced COX-2 expression. Exposure of cells with US results in IκBα phosphorylation in the cytosol and caused a marked IκBα degradation. In parallel to IκBα phosphorylation and degradation, US stimulation resulted in p65 translocation from cytosol to nucleus (Fig. 6D). Exposure of cells to US resulted in a 3-fold increase in κB-luciferase activity. Pretreatment of osteoblasts for 30 min with mAb against α5β1 or αvβ3 (20 μg/ml), rhodostomin (0.28 μM) or transfection with FAK(Y397F), p85(Δp85), Akt(K179A) or ERK(K52R) mutant for 24 hr markedly antagonized US-induced increase of κB-luciferase activity (Fig. 6E). These results indicate that NF-κB is the downstream effector for integrin/FAK/PI3K/Akt and ERK pathways in US-induced COX-2 expression.

Increase of COX-2 promoter activity by US stimulation

To further study the pathways involved in the action of US-induced COX-2 expression, transient transfection was performed using the mouse COX-2 promoter-luciferase constructs, pCOX2-Luc, which contains the mouse COX-2 gene between positions -370 and +70 fused to the luciferase reporter gene. Exposure to US led to a 3.5-fold increase in COX-2 promoter activity in osteoblasts. The increase of COX-2 activity by US stimulation was antagonized by mAb against α5β1, αvβ3 (20 μg/ml), rhodostomin (0.28 μM), LY294002 (10 μM), wortmannin (100 nM), PD 98059 (30 μM) and PDTC (30 μM) (Fig. 7A). In co-transfection experiments, the increase of COX-2 promoter activity by US was inhibited by the dominant negative

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mutant of FAK(Y397F), p85(Δp85), Akt(K179A) and ERK(K52R) (Fig. 7B). Taken together, these data suggest that the activation of integrin/FAK/PI3K/Akt/NF-κB pathway and ERK is required for the US-induced increase of COX-2 in osteoblasts.

US Stimulation enhanced bone nodule formation in osteoblasts

It has been reported that US enhanced the healing of bone fracture (Duarte, 1983; Wang et al., 1994). We then investigated whether FAK/PI3K/Akt pathway is involved in the differentiation of osteoblasts in response to long-term US stimulation. To clarify the involvement of FAK-PI3K-Akt pathway in the US-mediated bone nodule formation, FAK(Y397F), p85(Δp85) or Akt(K179A) mutants were transfected into MC3T3-E1 cells. After G418 selection, we isolated single clone of MC3T3/FAKDN, MC3T3/p85DN and MC3T3/AktDN, and vector control (MC3T3/vector). Formation of mineralized nodule is a marker for osteoblastic maturation. Alizarin red staining showed the mineralized nodules when osteoblasts were cultured in the medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 10 days (Fig. 8). MC3T3/vector cells exposed to US (20 min/day) increased bone nodule formation. However, transfection with dominant negative mutants of FAK, p85 and Akt attenuated US-induced bone nodule formation (Fig. 8). To confirm the crucial role of COX-2 in US-induced bone formation, selective COX-2 inhibitor NS-398 was used. Treatment of osteoblasts with NS-398 (0.3 μM) for 10 days antagonized US-induced bone nodule formation (Fig. 8). These data suggest that the activation of FAK/PI3K/Akt pathway is required for US to increase COX-2 expression and bone nodule formation in osteoblasts.

Discussion

Bone cells are equipped with mechanisms to sense diverse physical forces and transduce signals for adjustment of their microenvironment (Rubin et al., 1995). The noninvasive nature of US provides many advantages in practical applications. Although US is clinically used as a treatment for fracture repair, the molecular

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mechanisms by which US alters cell function or protein synthesis are virtually unknown. Here, we demonstrate that US exposure transiently increases the membrane expression of integrins and results in the expression of COX-2 and formation of PGE_2. Furthermore, long-term US treatment enhances the maturation of osteoblasts. The results of the present study suggest a role of integrin in the transduction of the acoustic pressure that leads to the expression of COX-2 and the enhanced maturation of osteoblasts.

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 (Hynes, 1992). We previously found that fibronectin fibrillogenesis is abundant underneath osteoblasts (Tang et al., 2003; 2004; 2005). α5β1 integrin is the classical fibronectin receptor and mediates critical interactions between osteoblasts and fibronectin and is required for bone morphogenesis and osteoblast differentiation (Moursi et al., 1997). Flow cytometry and immunofluorescence staining revealed that short-term US treatment transiently increased the cell surface expression of α5 and β1 integrins. Other integrins such as α2 and β3 are also transiently regulated by US treatment, suggesting that many kinds of integrins are probably involved in the regulation of osteoblastic function in response to US stimuli. Integrin-signalling pathways are involved in regulating the expression of a number of genes coding for substances involved in tissue repair, including cytokines and proteases (Arner et al., 1995). In the present study, we also show that US stimulation increased COX-2 expression and PGE_2 formation as detected by Western blotting analysis, qPCR and ELISA. Treatment with anti-α5β1, αvβ3 mAbs or rhodostomin (a RGD-dependent disintegrin that binds specifically to integrins αIIbβ3, α5β1 and αvβ3) antagonized the potentiating action of US stimulation on COX-2 expression, indicating that α5β1 and αvβ3 integrins are very important to mediate the action of US in osteoblasts. It has been found that cyclical pressure-induced strain results in rapid tyrosine phosphorglation of FAK, paxillin, and β-catenin in human articular chondrocytes (Lee et al., 2000). We here

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demonstrate that US stimulation increased phosphorylation of tyrosine 397 of FAK. Furthermore, the FAK(Y397F) mutant antagonized the US-mediated potentiation of COX-2 expression, suggesting that FAK activation is an obligatory event in US-induced COX-2 expression in these cells.

Increase of integrin expression may be related to the re-organization of actin cytoskeleton and cytoskeletal stiffness (Yang et al., 2005). The downstream signaling to integrin may involve the activation of several protein kinases such as Src (Parsons et al., 1997), PI3K (Naruse et al., 2000) or β-catenin (Lee et al., 2000). Furthermore, FAK contains tyrosine residues in motifs for binding to SH2 domain. Phosphorylated tyrosine 397 of FAK has been shown to serve as a binding site for the SH2 domain of the p85 subunit of PI3K (Chen et al., 1996). Phosphorylation of the p85 subunit is required for activation of the p110 catalytic subunit of PI3K. Pretreatment of osteoblast with PI3K inhibitors LY294002 and wortmannin antagonized the increase of COX-2 expression by US stimulation. This was further confirmed by the result that the dominant negative mutant of p85(Δp85) inhibited the enhancement of COX-2 promoter activity by US stimulation. The cytoplasmic serine kinase Akt was found to be activated by US stimulation in osteoblastic cells. These effects were inhibited by mAbs against α5β1 or αvβ3, rhodostomin or mutants of FAK and p85, indicating the involvement of integrin-FAK-PI3K-dependent Akt activation in US-mediated induction of COX-2. Activation of FAK-PI3K-Akt-dependent pathway has also been reported for β1 integrin to regulate cell survival in human lung fibroblast cells (Xia et al., 2004). In addition, regulation of angiogenesis by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor is also related to αvβ3 integrin/FAK/Akt signal cascade (Vincent et al., 2003). Take together, our results provide evidence that US up-regulates COX-2 in osteoblasts via the integrin/FAK/PI3K/Akt signaling pathway.

ERK also plays a critical role in integrin signaling (Kapur et al., 2003). We found that PD 98059 (a MEK inhibitor) also inhibited US-induced COX-2 expression. Furthermore, US-induced ERK activation was antagonized by mAb against α5β1 or αvβ3 integrin and FAK(Y397F) mutant but not by mutants of p85 and Akt, indicating

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that integrin and FAK but not PI3K and Akt might occur as the upstream molecules involved in US-induced activation of ERK. In mice and humans, the COX-2 promoter contains binding site for many transcription factors including NF-κB in the 5′ region of the COX-2 gene (Kim and Fischer, 1998). In addition, NF-κB has been shown to control the induced transcription of the COX-2 gene (Schmedtje et al., 1997). In this study, we demonstrate that US-induced COX-2 expression was inhibited by the NF-κB inhibitor, PDTC, indicating that activation of NF-κB might be involved in the induction of COX-2 protein caused by US stimulation. It has also been found that exposure of osteoblasts to US resulted in IκBα phosphorylation and IκBα degradation in the cytosol, the translocation of p65 from cytosol to nucleus. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that US increased NF-κB activity, which was inhibited by mAb against α5β1 or αvβ3 integrin, rhodostomin or DN mutants of FAK, p85, Akt or ERK. These results indicate that US might act through the integrin, FAK, PI3K, Akt and ERK pathway to induce NF-κB activation in osteoblasts.

Intracellular signals including NO, PGE_2, and calcium are involved in osteoblast differentiation and may occur in response to cellular homeostatic disturbance induced by US (Warden et al., 2001; Reher et al., 2002). It has been reported that US exposure increased PGE_2 release via up-regulation of COX-2 in osteoblast (Reher et al., 2002). We previously found that PGE_2 increased bone volume in tibia metaphysis of rats (Tang et al., 2005). In this study, we demonstrate that selective COX-2 inhibitor NS-398 inhibited US-induced bone nodule formation as detected by alizarin red staining. These data suggest that COX-2 and PGE_2 play a critical role in US-mediated bone nodule formation. However, the effect of US on the other prostaglandins except PGE_2 cannot be excluded. Increase of bone nodule formation by US stimulation was also antagonized by DN mutants of FAK, p85 or Akt, suggesting that the regulation of bone formation is parallel to the increase of COX-2 expression, and FAK/PI3K/Akt pathway is involved in the potentiating action of US.

In conclusion, the signaling pathway involved in US-induced COX-2 expression

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in rat osteoblasts has been explored. US stimulation increases COX-2 expression through the up-regulation of cell membrane integrins, activation of FAK, PI3K, Akt, ERK and NF-κB. Long-term US stimulation enhanced the differentiation of osteoblasts and bone nodule formation due to the increase of COX-2 expression.

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We thank Dr. H.R. Herschman for providing pCOX2-Luc; Dr. J.A. Girault for providing FAK(Y397F) mutant; Dr. R.H. Chen for providing p85 and Akt dominant negative mutants; Dr. M. Cobb for providing ERK2 dominant negative mutant.

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Footnotes

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Figure legends

Fig. 1 Increase of the cell surface expression of α5, β1 and β3 integrins by ultrasound stimulation.

MC3T3-E1 cells (A) and primary cultured osteoblasts (B) plated on six-well culture plates were exposed to ultrasound stimulation (US) for 20 min. The cells were then fixed and examined with flow cytometry at various time intervals after US stimulation. Note that expression of α5, β1, and β3 integrins in cell membrane transiently increased in response to US stimulation (n = 3, triplicate in each experiment). (C) Immunocytochemistry was performed and fluorescent images were obtained from confocal microscope. Compared with control, exposure to US markedly enhanced the clustering of β1 and β3 integrins 6 hr following US. (D) α2 but not α3 and α4 integrins increased after US stimulation. (E) Osteoblasts were cultured on plates pre-coated with fibronectin, vitronectin and collagen (10 μg/ml, 500 μl for each) for 24 hr and cells were then exposed to US for 20 min. The integrin expression of the cells were examined with flow cytometry 6 hr after US stimulation. Data are presented as mean ± SE. (n=3)

Fig. 2 Increase of COX-2 expression by US stimulation in cultured osteoblasts.

(A) MC3T3-E1 or primary osteoblasts were exposed to US for 20 min. Protein levels of both COX-1 and COX-2 were measured 1, 3, 6, 12 and 24 hr after US treatment. (B) The cells were stimulated with US for 20 min, and the mRNA for COX-2 was then analyzed by qPCR at various time intervals. Note that US enhanced protein and mRNA levels of COX-2 in osteoblasts. (C) PGE_2 levels in supernatant of cultured osteoblasts with and without US treatment were determined by ELISA. Data are presented as mean ± SE. *, $p<0.05$ as compared with control (n=3).

Fig. 3 Integrin and FAK are involved in US-mediated increase of COX-2

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expression.

(A) Osteoblasts were pretreated with mAb against α5β1 or αvβ3 integrin (20 μg/ml), rhodostomin (Rn, 0.28 μM) for 30 min or transfected with FAK(Y397F) mutant for 24 hr followed by exposure to US for 20 min. COX-2 protein expression was determined by immunoblotting 24 hr following US. The quantitative data were shown in the lower panel (n=3). (B) Osteoblasts were stimulated with US for 20 min, and then phosphorylated FAK was determined at various time intervals after US stimulation. (C) Cells were pretreated with mAb against α5β1 or αvβ3 integrin (20 μg/ml), rhodostomin (0.28 μM) for 30 min or transfected with FAK(Y397F) mutant for 24 hr followed by exposure to US for 20 min. FAK phosphorylation was determined 10 min after US stimulation. Protein level of FAK was shown for comparison. Data are presented as mean ± SE. *, $p<0.05$ as compared with control. #, $p<0.05$ as compared with US-treated group.

Fig. 4 Involvement of PI3K signaling pathway in response to US stimulation in osteoblast.

(A) Osteoblasts were stimulated with US for 20 min, and cell lysates were immunoprecipitated (IP) with an antibody specific for p85. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted (WB) with anti-phosphotyrosine (PY) at various time intervals after US stimulation. (B) Osteoblasts were pretreated with mAb against α5β1 or αvβ3 integrin (20 μg/ml), rhodostomin (Rn, 0.28 μM) for 30 min or transfected with FAK(Y397F) mutant or pcDNA3 (vector) for 24 hr followed by exposure to US for 20 min, and cell lysates were immunoprecipitated with an antibody specific for p85. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine 15 min after US stimulation.

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Fig. 5 PI3K and Akt are involved in the potentiation of COX-2 expression by US stimulation.

(A) Osteoblasts were pretreated with PI3K inhibitors of LY294002 (LY) and wortmannin (WM) for 30 min or transfected with p85 and Akt dominant negative mutants (DN) for 24 hr followed by stimulation with US for 20 min, and COX-2 expression was determined by immuoblotting 24 hr following US. The quantitative data were shown in the lower panel (n=3). (B) Osteoblasts were stimulated with US for 20 min, and then Akt phosphorylation was determined at various time intervals after US stimulation. (C) Cells were pretreated with mAb against $\alpha5\beta1$ or $\alpha v\beta3$ integrin (20 μg/ml), rhodostomin (Rn, 0.28 μM) for 30 min or transfected with FAK(Y397F), p85(Δp85) mutants or pcDNA3 (vector) for 24 hr followed by exposure to US for 20 min, and Akt phosphorylation was then determined 15 min after US stimulation. The protein level of Akt was shown for comparison. Data are presented as mean ± SE. *, $p<0.05$ as compared with control. #, $p<0.05$ as compared with US-treated group.

Fig. 6 Involvement of ERK and NF-κB in US-mediated COX-2 expression in osteoblasts.

(A) Osteoblasts were pretreated with PD 98059 or PDTC for 30 min followed by stimulation with US for 20 min, and COX-2 expression was determined by immuoblotting 24 hr following US. The quantitative data are shown in the lower panel. (B) Osteoblasts were treated with US for 20 min, and then ERK 1/2 phosphorylation was determined at various time intervals after US stimulation. (C) Cells were pretreated with mAb against $\alpha5\beta1$ or $\alpha v\beta3$ (20 μg/ml) for 30 min or transfected with FAK, p85, Akt DN mutants or pcDNA3 (vector) for 24 hr followed by exposure to US for 20 min, and ERK 1/2 phosphorylation was then determined 15 min after US stimulation. (D) Cells were treated with US for 20 min, and cytosolic phosphorylated or

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unphosphorylated IκBα was determined at various time intervals after US stimulation. In addition, the levels of p65 in the cytosol and nucleus were also measured as well. (E) Osteoblasts were transfected with κB-luciferase and then pretreated with mAb against α5β1 or αvβ3 integrin (20 μg/ml), rhodostomin (0.28 μM) for 30 min or transfected with DN mutant of FAK, p85, Akt or ERK2 for 24 hr. κB-luciferase activity was measured 24 hr after US stimulation, and the results were normalized to the β-galactosidase activity and expressed as the mean ± SE. for three independent experiments performed in triplicate. *, $p<0.05$ as compared with control. #, $p < 0.05$ as compared with US-treated group.

Fig 7. The signaling pathways involved in the increase of COX-2 promoter activity by US stimulation.

(A) The COX-2 promoter activity was evaluated by transfection with the pCOX2-Luc luciferase expression vector. Osteoblasts were pretreated with mAb against α5β1 or αvβ3 integrin (20 μg/ml), rhodostomin (0.28 μM), LY294002 (10 μM), wortmannin (100 nM), PD 98059 (30 μM) or PDTC (30 μM) for 30 min before stimulation with US. (B) Cells were co-transfected with pCOX2-Luc and the DN mutant of FAK, p85, Akt or ERK2 and then treated for 20 min with US. Luciferase activity was measured 24 hr after US stimulation, and the results were normalized to the β-galactosidase activity and expressed as the mean ± SE. for three independent experiments performed in triplicate. *, $p<0.05$ as compared with control. #, $p < 0.05$ as compared with US-treated group.

Fig 8. FAK-PI3K-Akt signaling pathway is involved in the enhancement of mineralization by long-term ultrasound stimulation in MC3T3-E1 cells.

(A) MC3T3/vector, MC3T3/FAKDN, MC3T3/p85DN and MC3T3/AktDN cells were plated in 6-wll plates and cultured in medium containing vitamin

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C (50 μg/ml) and β-glycerophosphate (10 mM) and the cells were exposed to US for 20 min/day. On Day-10, cultures were fixed in 75% ethanol, and mineralized nodule formation was assessed by alizarin red-S staining. (B) The bound staining was eluted with solution of 10% cetylpyridinium chloride and quantified using a microplate reader. Note that long-term US stimulation increased the mineralization and transfection with DN mutant of FAK, p85 or Akt or treatment with NS-398 antagonized the potentiating action of US. Data are presented as mean ± SE (n = 4). *, $p<0.05$ as compared with control. #, $p<0.05$ as compared with US-treated group.

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Figure 1

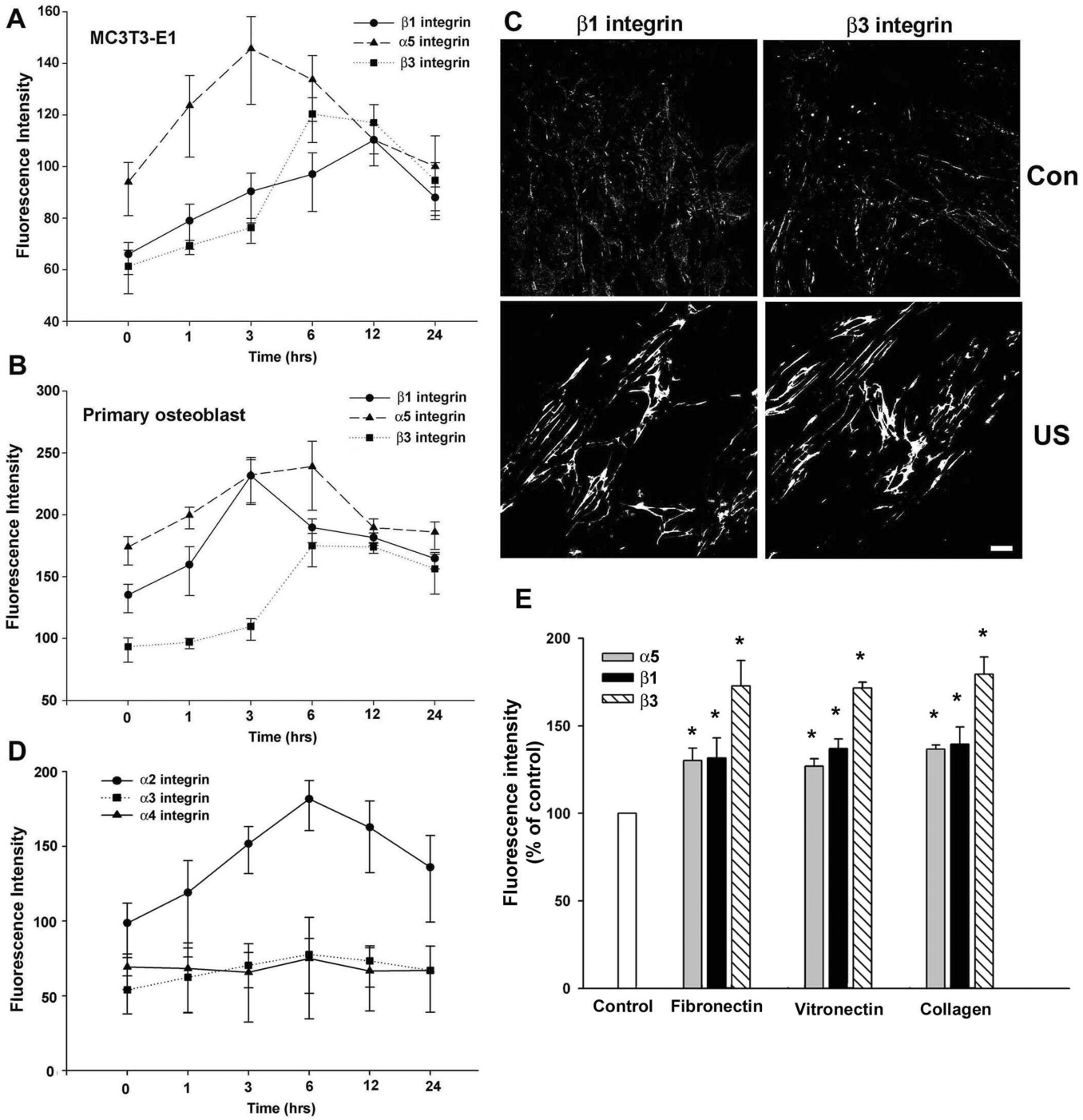

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Figure 2

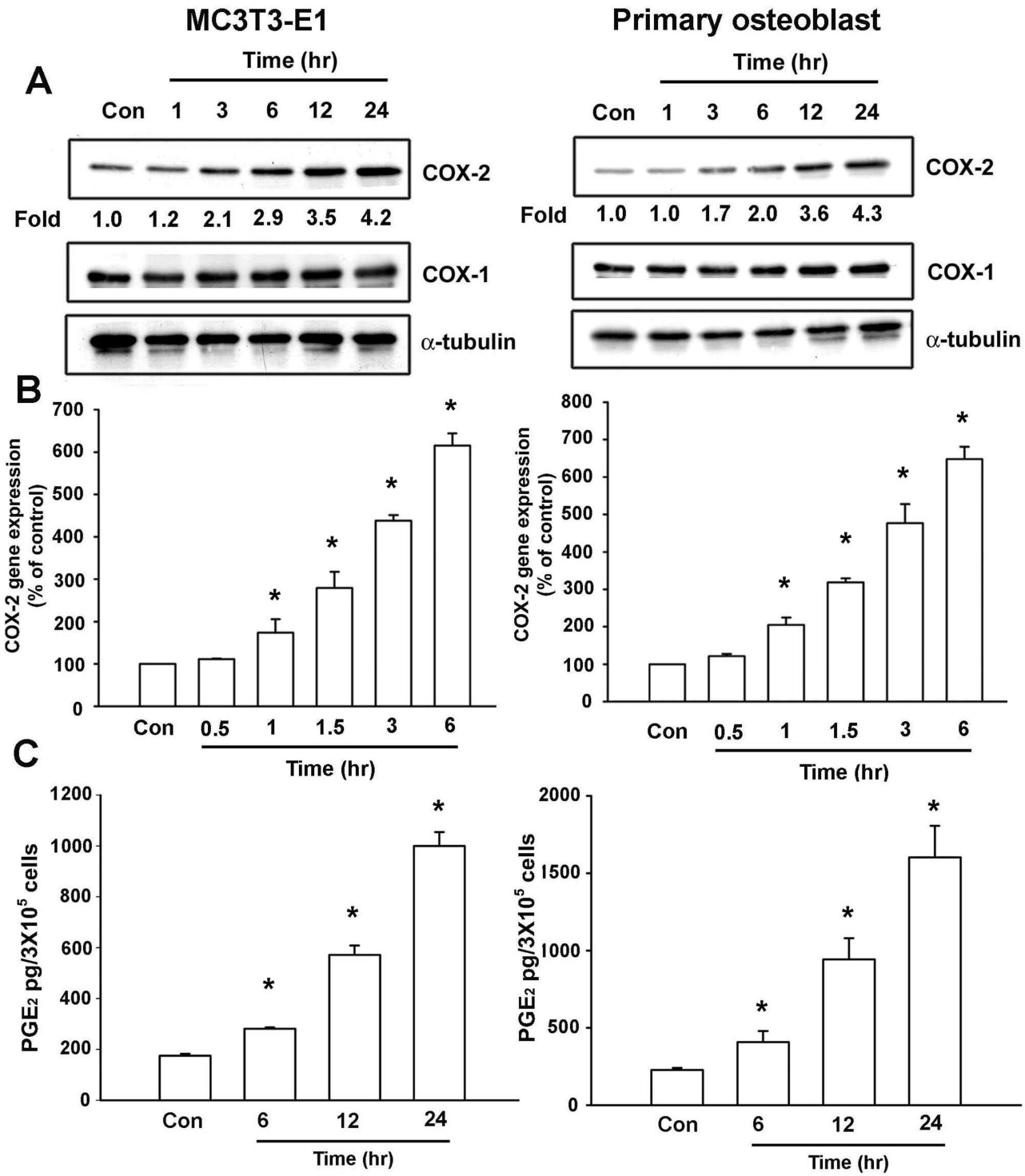

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Figure 3

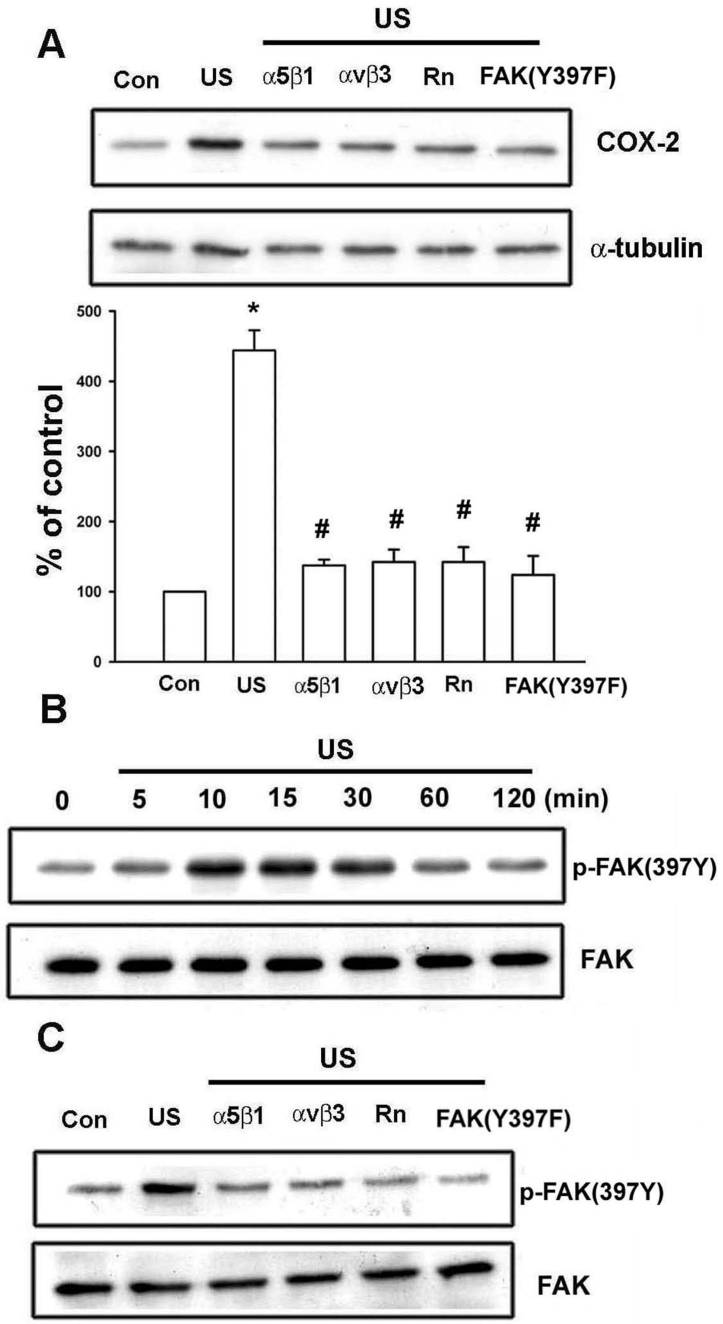

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Figure 4

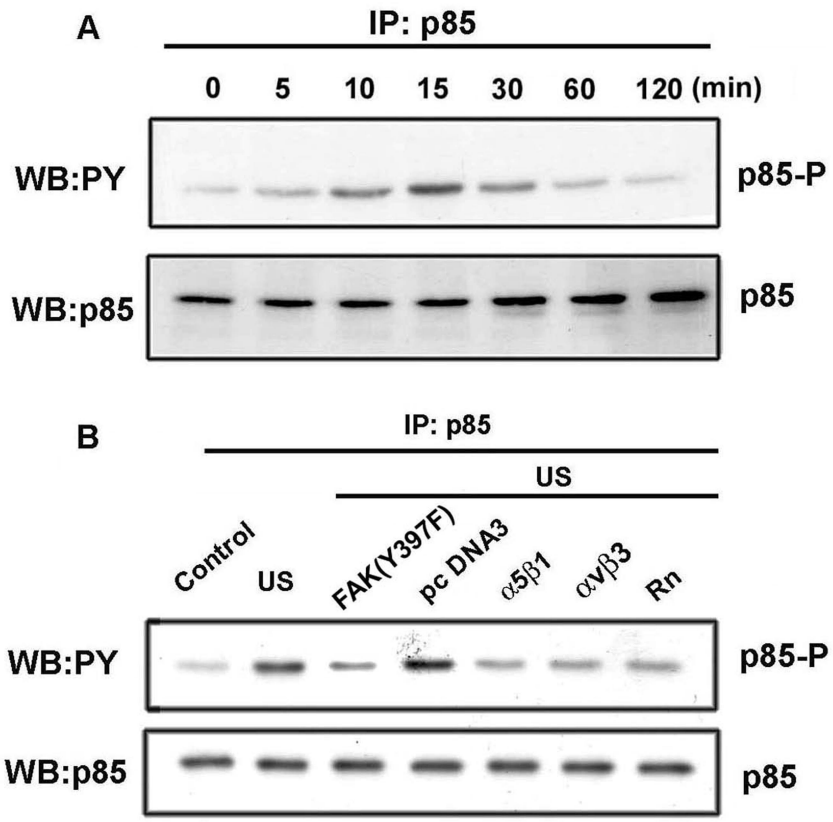

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Figure 5

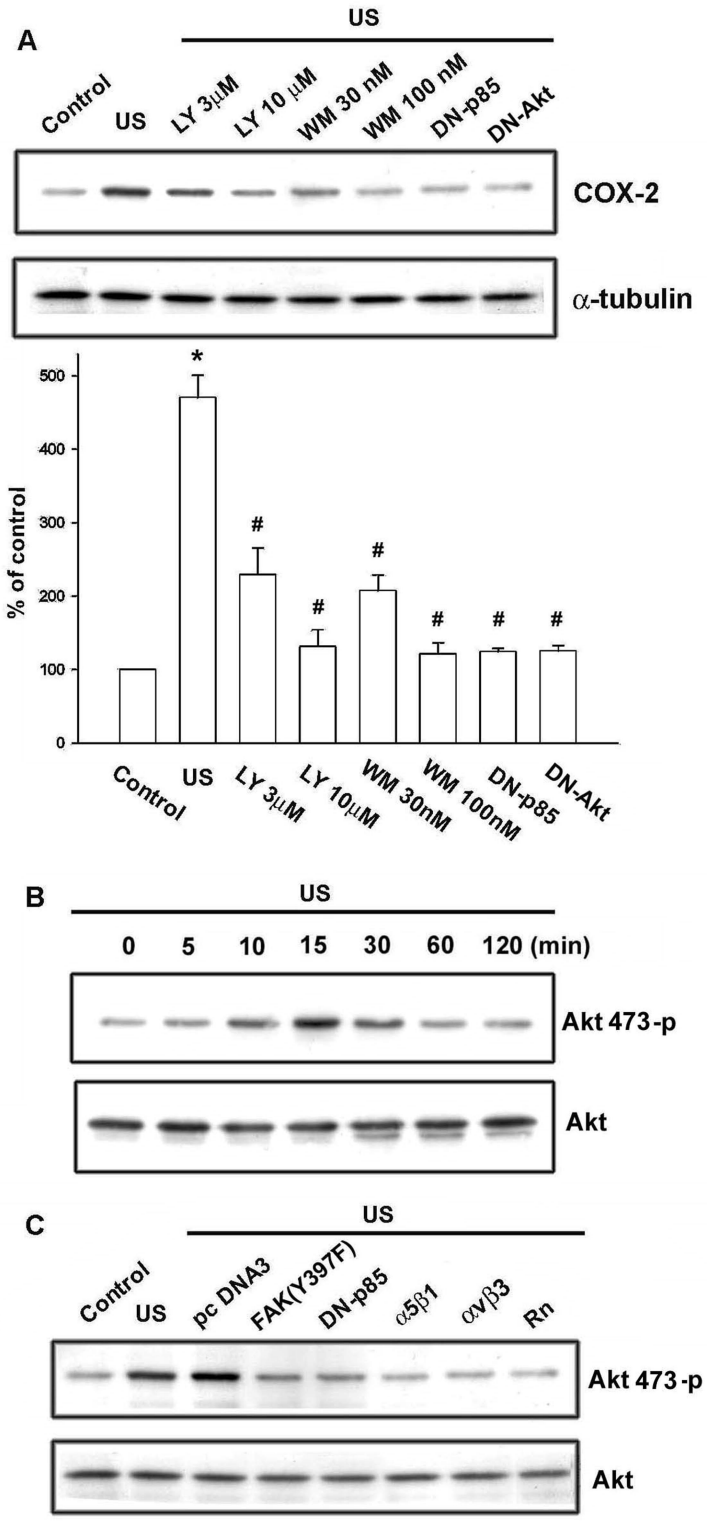

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Figure 6

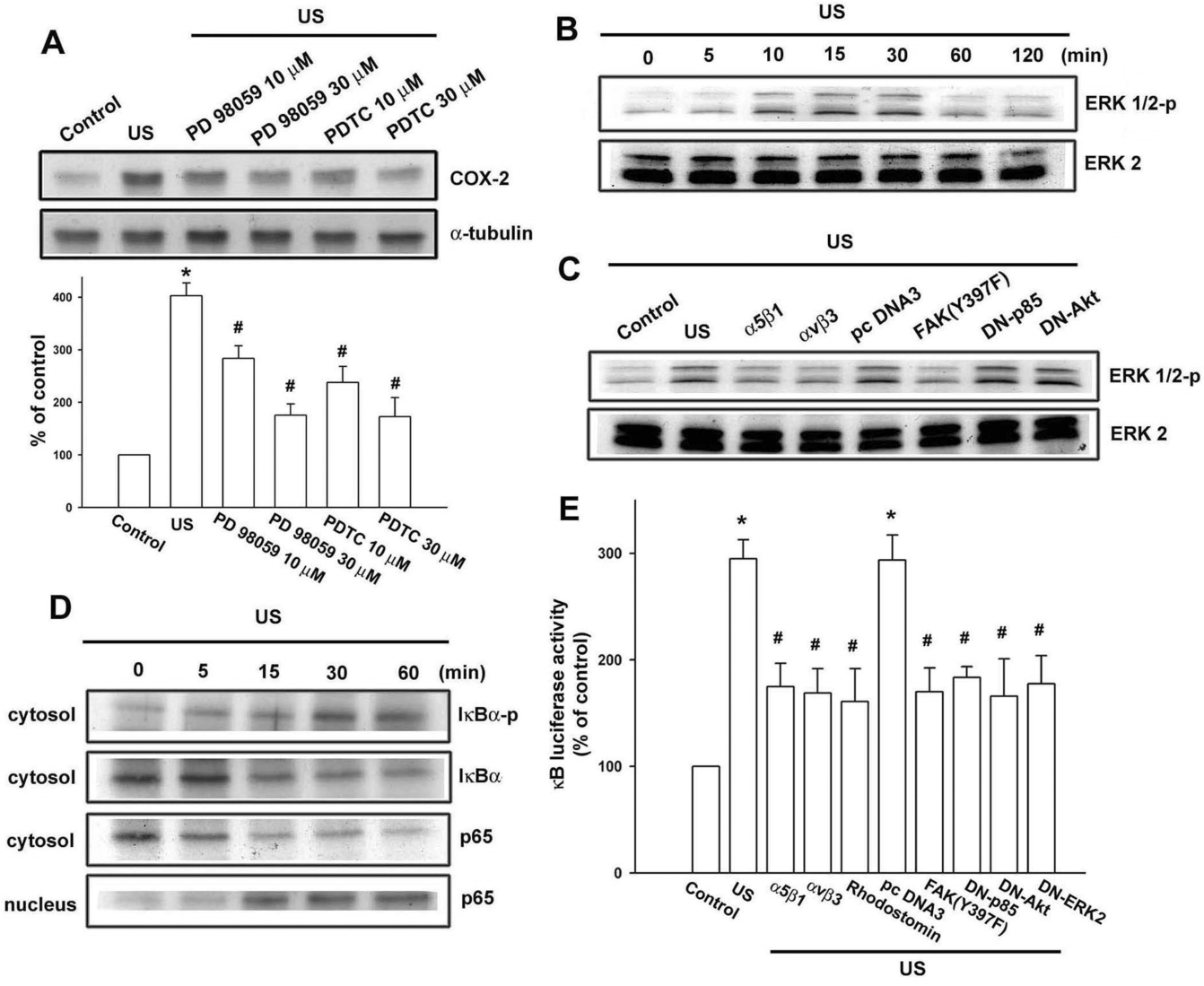

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Figure 7

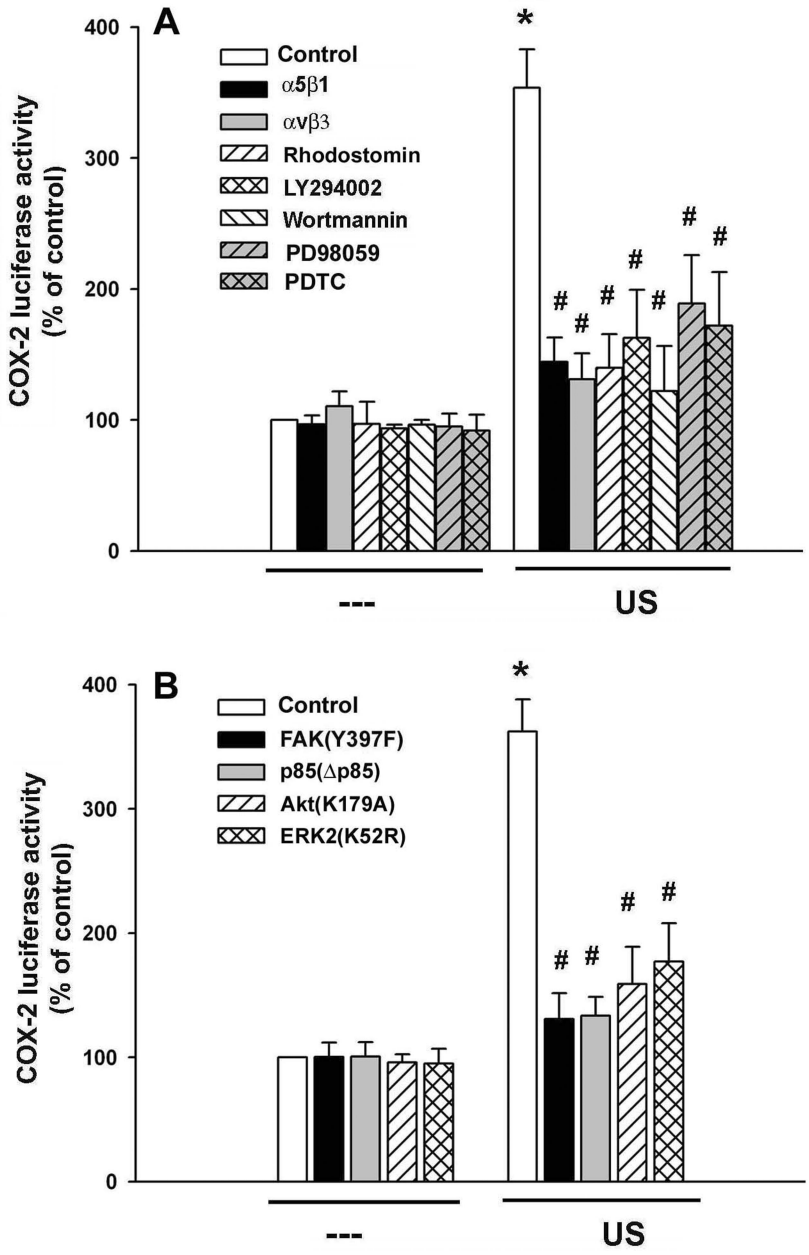

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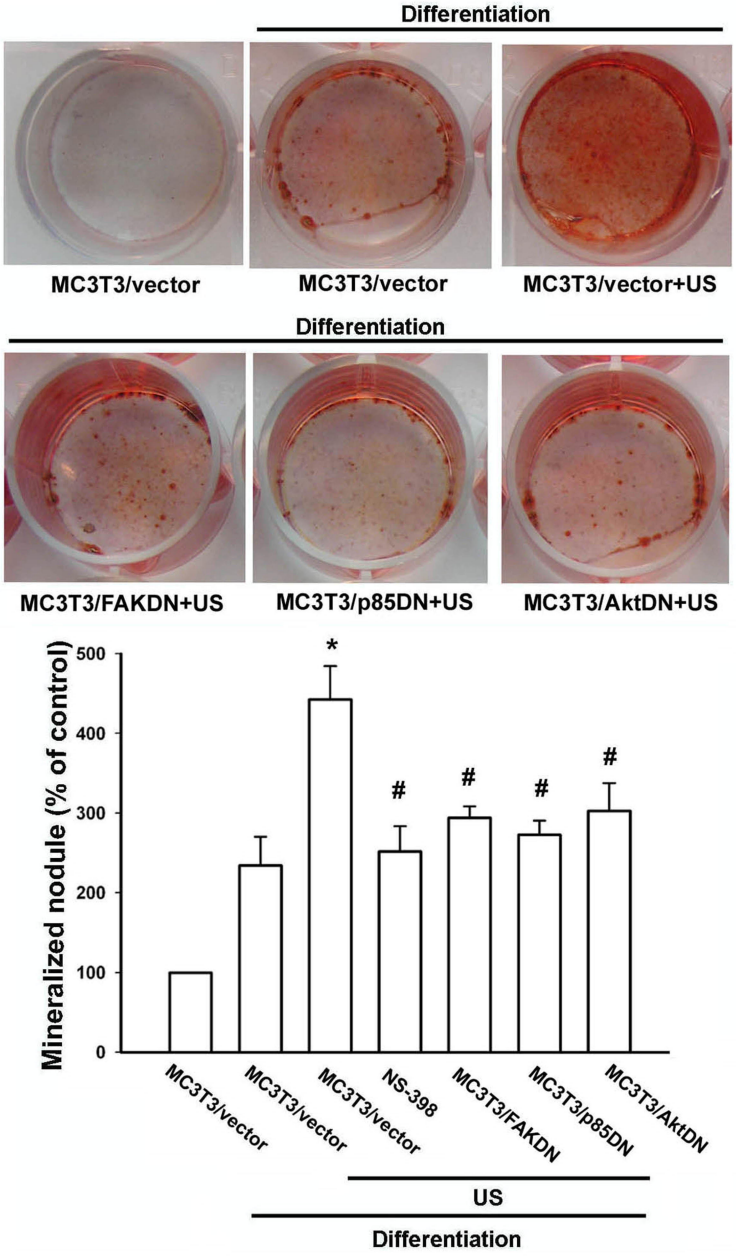
Figure 8



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