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Molecular Mechanisms of Low Intensity Pulsed Ultrasound in Human Skin Fibroblasts*

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Soluble factors such as polypeptide growth factors, mitogenic lipids, inflammatory cytokines, and hormones are known regulators of cell proliferation. However, the effect of mechanical stimuli on cell proliferation is less well understood. Here we examined the effect of low intensity pulsed ultrasound (US), which is used to promote wound healing, on the proliferation of primary human foreskin fibroblasts and the underlying signaling mechanisms. We show that a single 6–11-min US stimulation increases bromodeoxyuridine incorporation. In addition, an increase in the total cell number is observed after sequential US stimulation. US induced stress fiber and focal adhesion formation via activation of Rho. We further observed that US selectively induced activation of extracellular signal-regulated kinase (ERK) 1/2. Inhibition of Rho-associated coiled-coil-containing protein kinase (ROCK) prevented US-induced ERK1/2 activation, demonstrating that the Rho/ROCK pathway is an upstream regulator of ERK activation in response to US. Consequently, activation of ROCK and MEK-1 was required for US-induced DNA synthesis. Finally, an integrin β_1 blocking antibody as well as a RGD peptide prevented US-induced DNA synthesis. In addition, US slightly increased phosphorylation of Src at Tyr^{416}, and Src activity was found to be required for ERK1/2 activation in response to US. In conclusion, our data demonstrate for the first time that US promotes cell proliferation via activation of integrin receptors and a Rho/ROCK/Src/ERK signaling pathway.

Signal transduction mechanisms of receptor tyrosine kinase or heptahelical receptors have been studied extensively over the last years. However, the effect of acoustic pulsed energy on cell growth and the signal transduction mechanisms induced by this type of mechanical stimulation are not well understood. Previous studies have shown that mechanical stress such as stretch and shear stress can induce DNA synthesis in certain cell types and that extracellular signal-regulated kinase (ERK)[1] 1/2 can be activated by mechanical stress (1–4).

Low intensity pulsed ultrasound (US) is a special type of acoustic pulsed energy that is increasingly used as a supplementary therapy to promote bone and wound healing (5). US, transmitting as an acoustic pressure wave and applying mechanical stress indirectly to the tissues, has been reported to promote osteogenesis and protein synthesis, calcium uptake, and DNA synthesis in different cells (6–9). US-induced DNA synthesis seems cell type-dependent; US promotes DNA synthesis in human osteoblasts, gingival fibroblasts, and periosteal cells (6, 8, 10), but not in chondrocytes (11, 12). However, the molecular mechanisms by which US induces DNA synthesis or even cell proliferation are largely unknown.

There is growing evidence that integrins are promising candidates for sensing extracellular matrix-derived mechanical stimuli and converting them into biochemical signals (13, 14). Integrin-associated signaling pathways include an increase in tyrosine phosphorylation of several signaling proteins, activation of serine/threonine kinases, and alterations in cellular phospholipid and calcium levels (15, 16). These events are associated with the formation of focal adhesions, which contain structural proteins such as talin and vinculin, the signaling molecules focal adhesion kinase and Src, and the adaptor proteins paxillin, p130^{CAS}, and Shc. Focal adhesions act as a bridge to link integrin cytoplasmic domain to the cytoskeleton and activate integrin-associated signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway (17) and the Rho pathway (18, 19). Rho and its downstream target Rho kinase/Rho-associated coiled-coil-containing protein kinase (ROCK) (20) are involved in the reorganization of cytoskeletal components (21, 22) as well as cell cycle progression (1, 23, 24). Integrin-dependent adhesion can also trigger ligand-independent epidermal growth factor receptor (EGFR) activation to transduce downstream signaling (25).

In the present study, we examined the effect of US on cell proliferation and the signaling mechanism mediating this effect in primary human foreskin fibroblasts. We report that US activates ERK1/2 by a ROCK-dependent mechanism. Both ERK1/2 and ROCK are required for US-induced cell proliferation. Furthermore, integrins were found to be responsible for US-induced cell proliferation. We suggest that integrins act as mechanotransducers to transmit acoustic pulsed energy into intracellular biochemical signals inducing cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—The materials were purchased from the following sources: anti-phospho-p44/42 (Thr^{202}/Tyr^{204}) antibody (E10), anti-p42 MAPK antibody (3A7), anti-phospho-EGFR (Tyr^{1068}) antibody, anti-EGFR

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[1] The abbreviations used are: ERK, extracellular signal-regulated kinase; US, low intensity pulsed ultrasound; MAPK, mitogen-activated protein kinase; ROCK, Rho-associated coiled-coil-containing protein kinase; FCS, fetal calf serum; PBS, phosphate-buffered saline; MEK, MAPK-ERK kinase; BrdU, 5-bromo-2′-deoxyuridine; EGFR, epidermal growth factor receptor; PI, phosphatidylinositol.

polyclonal antibody and the MEK1 inhibitor PD98059 from Cell Signaling Technology (Beverly, MA); 5-bromo-2′-deoxyuridine (BrdU), lysophosphatic acid, and myelin-binding protein peptide (APRTPGGRR) from Sigma; Rho activation assay kit, anti-phospho-Src (Tyr^{416}) antibody (9A6), anti-Src antibody (GD11), p42 MAPK polyclonal antibody, and anti-paxillin antibody (5H11) from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-BrdU antibody (Bu20a) and horseradish peroxide-conjugated secondary antibodies from DAKO (Hamburg, Germany); the Src family inhibitor PP1 and the EGFR tyrosine kinase inhibitor AG1478 from Calbiochem-Novachem (La Jolla, CA); anti-integrin β_1 antibody (P4C10) from Chemicon International (Temecula, CA); H-Arg-Gly-Asp-OH peptide (RGD) from Bachem AG (Bubendorf, Switzerland); the ROCK inhibitor Y27632 from Tocris Cookson (Ellisville, MO); CytoTox 96® nonradioactive cytotoxicity assay kit from Promega (Madison, WI); Alexa-phalloidin from Molecular Probes (Eugene, OR); biotin-tyramid signal amplification (TSA) reagent from PerkinElmer Life Sciences; Texas Red streptavidin from Jackson ImmunoResearch Laboratories (West Grove, PA); and polyvinylidene difluoride membrane from Roche Applied Science.

Cell Isolation and Culture—Primary skin fibroblasts were isolated by the outgrowth method using foreskin tissues. Full-thickness foreskin tissues were placed directly in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) supplemented with 20% fetal calf serum (FCS), 2 mM L-glutamine and then cut into small pieces of 0.5–1 mm^3 using a scalpel under sterile conditions. The small tissue pieces were seeded in 6-well plates (5–10 pieces/well) and cultured in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 125 ng/ml amphotericin B at 37 °C in a 5% CO_2 humidified atmosphere. The medium was changed after 12 and 24 h; the small tissue pieces were then transferred to new culture plates. During the next 3–5 days, the cells grew out from the tissue pieces and gradually formed a confluent layer. After reaching confluence, the cells were subcultured by trypsinization using a 0.25% trypsin solution containing 0.01% EDTA in phosphate-buffered saline (PBS). The medium was changed the day after seeding and each second or third day thereafter. The experiments were performed using cell populations between passages 2 and 10.

Stimulation with US—To stimulate the cultured cells with US, a modified SAFHS® apparatus (Model 2A, Exogen Inc., Piscataway, NJ) was used, which produces a 1.5-MHz ultrasound wave, 200-μs pulse modulated at 1 kHz, with an output intensity of 30 mW/cm^2. To enable cultured cells to be treated by ultrasound, six ultrasound transducers are fitted on a plastic frame and connected to the control panel of the signal generator via six independent cables. Therefore, the cells were seeded in 6-well plates for all the experiments. The operation of the transducers was checked before each experiment. The plates were placed on ultrasound transducers using a coupling gel. The untreated plates were always put in a separate incubator for control purposes.

BrdU Incorporation Assay—BrdU incorporation was visualized by immunostaining as described (26). Briefly, the cells were cultured in 6-well plates and serum-starved for 24–36 h. 6 h after stimulation with US, 50 μM BrdU in Dulbecco's modified Eagle's medium was added to the cultures. After additional incubation for 18 h, the cells were washed with PBS and then fixed with ice-cold ethanol/acetic acid (95/5, v/v) at 4 °C. Thereafter 0.05 M HCl was added to the fixed cells (4 °C for 20 min). After washing in PBS with 0.05% Tween 20 (PBST), DNA was cleaved by incubation for 45 min at 80 °C with formamide/trisodium citrate (88 mg of trisodium citrate in 38 ml of formamide). Nonspecific binding was blocked by incubation with serum (diluted 1:1 with PBS). Mouse anti-BrdU diluted 1:200 in Tris-buffered saline containing bovine serum albumin (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% albumin bovine fraction V) was added and incubated with gentle agitation for 1 h at room temperature. Rabbit anti-mouse horseradish peroxidase (1:100 in Tris-buffered saline containing bovine serum albumin) was added and incubated for 2 h. Finally, after washing, the cells were stained using diaminobenzimidine tetrahydrochloride dihydrate as substrate and 0.3% hydrogen peroxide with nickel- and cobalt-chloride as intensifier. Total cell number and BrdU-positive nuclei were counted. BrdU incorporation presented as the percentage of positive nuclei to total cell number.

Total Cell Number Assay—The total cell number for each sample was estimated by quantitation of lactate dehydrogenase after cell lysis using the CytoTox 96® nonradioactive cytotoxicity assay kit according to the manufacturer instructions.

Western Blotting—Skin fibroblasts were lysed at 4 °C in 250 μl of solution containing 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.2 mM Na_3VO_4, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). The lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4 °C. The lysate (30–40 μg) was mixed with 2× Laemmli reducing sample buffer and heated at 95 °C for 5 min. The proteins were separated by SDS-PAGE on 10% gel and then transferred to polyvinylidene difluoride membrane. Nonspecific binding was blocked with 5% bovine serum albumin in PBST for 2 h at room temperature. The blots were incubated with phospho-p42/44 MAPK (Thr^{202}/Tyr^{204}) monoclonal antibody, phospho-EGFR (Tyr^{1068}) monoclonal antibody, or phospho-Src (Tyr^{416}) monoclonal antibody overnight at 4 °C. After further incubation with horseradish peroxidase-conjugated secondary antibody for 1 h, the bound antibodies were detected with an enhanced chemiluminescence detection kit. The blots were then stripped and reprobed with P42 MAPK monoclonal antibody, EGFR polyclonal antibody, and Src polyclonal antibody for total ERK1/2, EGFR, and Src, respectively.

Immune Complex Kinase Assay for p42/44 MAPK—Quiescent and confluent skin fibroblasts were lysed as described above. Immunoprecipitation was performed using a polyclonal anti-p42 MAPK antibody. The samples were incubated on a rotator for 2 h at 4 °C. Protein A-Sepharose beads (50 μl, 1:1 slurry) were added for the second hour. Immune complexes were collected by centrifugation and washed twice in lysis buffer and twice in kinase buffer (15 mM Tris-HCl, pH 7.4, 15 mM $MgCl_2$). The kinase reaction was performed by resuspending the pellet in 25 μl of kinase assay mixture containing kinase buffer, 100 μM ATP, 100 μCi/ml [γ-^{32}P]ATP, and 0.5 mg/ml myelin-binding protein peptide (APRTPGGRR) for the p42 MAPK assays. Incubations were performed for 10 min (linear assay conditions) at 30 °C and terminated by spotting 20 μl of the supernatant onto P81 chromatography paper (Whatman). The filters were washed four times for 5 min in 0.5% orthophosphoric acid, immersed in acetone, and dried before Cerenkov counting. The specific activity of [γ-^{32}P]ATP used was 900–1200 cpm/pmol.

Rho Activation Assay—The activation of Rho was determined by affinity precipitation using a Rho activation assay kit. The skin fibroblasts were lysed with Mg^{2+} lysis/wash buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM sodium fluoride, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C. Aliquots of the cell lysates (1 mg of protein in 1 ml) were mixed with 30 μl of the rhotekin RBD-agarose slurry, and the mixture was rotated for 45 min at 4 °C. The beads were washed three times with 1 ml of Mg^{2+} lysis/wash buffer. The beads were dried with a Hamilton syringe and resuspended in 40 μl of 2× Laemmli reducing sample buffer, and the suspension was heated for 5 min at 95 °C. The proteins were separated on 12.5% SDS-polyacrylamide gel electrophoresis and detected by Western blotting using 3 μg/ml of monoclonal anti-Rho. Loading controls were taken from each lysate sample prior to affinity precipitation.

Immunofluorescence—Serum-starved cells were preincubated with the ROCK inhibitor Y27632 (20 μM) or the MEK1 inhibitor PD98059 (20 μM) for 1 h and stimulated by US for 11 min. The cultures were stopped at 45 min after US and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Nonspecific binding was blocked with FCS in PBS (1:1) for 30 min. After permeabilization with 0.2% Triton X-100 in PBS for 10 min, the cells were then incubated with monoclonal anti-paxillin (5H11) (1:100, 1 h), horseradish peroxidase-anti-mouse IgG (1:100, 1 h), biotin-tyramid signal amplification reagent (1:500, 15 min), and streptavidin-Texas Red (1:200, 30 min). Thereafter, F-actin was labeled with Alexa-phalloidin 488 (2.5 unit/ml) for 30 min. The nuclei were stained with Hoechst 33258. Tris-buffered saline containing bovine serum albumin buffer was used for dilution. The cells were thoroughly washed after each step in PBST buffer three times each for 5 min. Digital fluorescence images were obtained with an Olympus microscope.

Statistical Analysis—The data were presented as the means ± S.E. Statistical significance was evaluated using one-way analysis of variance for comparison between the control and test groups. The values were considered to be statistically different when $p < 0.05$.

RESULTS

US Induces DNA Synthesis and Cell Proliferation in Human Skin Fibroblasts—Fibroblasts are the major cell type in connective tissue and are critical for tissue restoration and remodeling after injury. Therefore, we used primary skin fibroblasts as a model to understand the mechanism of US to promote tissue repair. To examine the effect of US on DNA synthesis, the cells were cultured in the absence or presence of 5% FCS

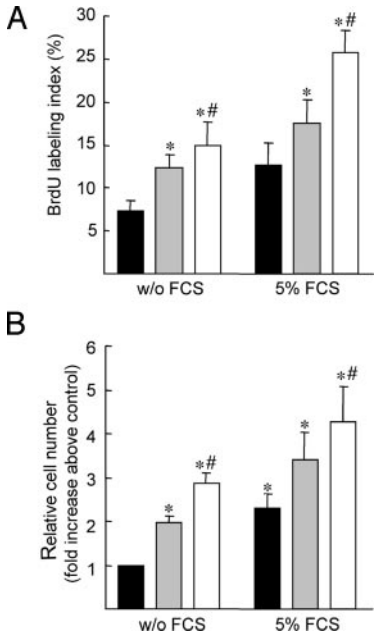


FIG. 1. **US induces increase in cell proliferation of human skin fibroblasts.** *A*, skin fibroblasts were serum-starved for 24 h and left them without US stimulation (*black bars*) or stimulated with US for 6 min (*shaded bars*) or 11 min (*white bars*) in the absence or presence of 5% FCS. BrdU (50 μM) was added to the cultures 6 h after US. The cultures were stopped after an additional incubation for 18 h. The quantitation of BrdU incorporation into newly synthesized DNA of proliferating cells was performed by immunostaining against BrdU, and the BrdU labeled nuclei were visualized by 3,3′-diaminobenzidine tetrahydrochloride dihydrate. The data shown are expressed as the percentages of BrdU-positive nuclei in three independent microscopic fields per condition and are the means ± S.E. *, $p < 0.05$ compared with without US-stimulated control cells; #, $p < 0.05$ compared with cells stimulated by US for 6 min. *B*, subconfluent skin fibroblasts were serum-starved overnight and cultured in medium without or with 5% FCS. The cells were untreated or exposed to 6 or 11 min US, once a day for 7 days. The cultures were stopped 24 h after the last US application (day 8), and the total cell lysates were collected with 0.9% Triton X-100. Quantitative measurement of lactate dehydrogenase in cell lysates was performed with the CytoTox 96 assay. The data shown are the fold increases above control (w/o FCS and US) in three independent wells and are the means ± S.E. *, $p < 0.05$ compared with control cells; #, $p < 0.05$ compared with cells stimulated by US for 6 min.

and stimulated with US for 6 min or 11 min, and BrdU incorporation was determined after 24 h of incubation. As shown in Fig. 1*A*, treatment with US for 6 or 11 min significantly increased the BrdU labeling index both in the absence and the presence of 5% FCS. Next, we examined the effect of US on actual cell proliferation by quantifying the total cell number using the CytoTox 96® assay. In accordance with US-induced DNA synthesis, seven sequential US stimulations (once per day for 6 or 11 min over 7 days) significantly increased the total cell number in the absence of FCS by 2- and 2.8-fold, respectively, compared with control (Fig. 1*B*, *left columns*). Even in the presence of 5% FCS, US induced 1.48-fold (6 min) and 1.7-fold (11 min) increases of cell number (Fig. 1*B*, *right columns*). Both the BrdU incorporation assay and total cell number analysis showed that the effect of 11 min of US stimulation on cell proliferation was more pronounced than 6 min of stimulation. Therefore, 11 min of US stimulation was used in all of the subsequent experiments.

US Induces Rho-mediated Stress Fiber and Focal Adhesion Formation—Mechanical stimulation may induce formation of actin stress fibers and focal adhesions (27). Therefore, we examined whether US has any effect on the organization of the actin cytoskeleton, in particular, on stress fiber and focal adhesion formation. Actin was diffusely distributed throughout the cytoplasm and formed a thin cortical ring in control skin fibroblasts. Paxillin was also distributed throughout the cytoplasm (Fig. 2*A*, *panels a*, *e*, and *i*). 30 and 60 min after US, we observed an intense formation of actin stress fibers, and paxillin immunofluorescence was strikingly redistributed by US to focal adhesions with similar kinetics (Fig. 2*A*, *panels b*, *f*, and *j* and data not shown).

The small G-protein Rho and its downstream target Rho kinase/ROCK modulate the organization of actin stress fibers and the formation of focal adhesions as well as the redistribution of cytoskeletal components (21, 22). To examine the role of Rho/ROCK in US-induced stress fiber and focal adhesion formation in primary skin fibroblasts, the cells were treated with 20 μM of the selective ROCK inhibitor Y27632 (28) for 60 min and subsequently analyzed by indirect immunofluorescence. Y27632 completely prevented both US-induced stress fiber formation and focal adhesion assembly (Fig. 2*A*, *panels d*, *h*, and *i*), indicating that Rho/ROCK plays a pivotal role in US-stimulated stress fiber and focal adhesion formation. Using a rhotekin RBD affinity precipitation assay (29), we demonstrate that US induces a strong activation of RhoA, which was comparable with that induced by the bioactive lipid lysophosphatic acid, a potent activator of RhoA (30).

The Rho/ROCK Pathway Is Involved in US-induced DNA Synthesis—The Rho/ROCK signaling pathway has also been implicated in the G_1-S phase progression of the cell cycle (1, 23, 24). Therefore, we next investigated whether activation of the Rho pathway could be involved in US-stimulated DNA synthesis. As shown in Fig. 2*B*, BrdU incorporation in response to US was substantially inhibited in the presence of Y27632. Thus, activation of Rho/ROCK is required for DNA synthesis induced by US stimulation.

US-induced ERK1/2 Activation Mediates DNA Synthesis—The ERK cascade is a potent mediator of cell cycle progression in response to a variety of stimuli. Indeed, ERK1/2 phosphorylation slightly increased directly at the end of US stimulation, peaked between 10 and 20 min (2.26 ± 0.41-fold of control) after US stimulation, and gradually decreased to base-line levels within 60 min (Fig. 3*A*). There was no change in the total ERK1/2 protein content (Fig. 3*A*). In addition to Western blotting analysis of ERK1/2 phosphorylation, we performed ERK *in vitro* kinase assays in primary skin fibroblasts. As shown in Fig. 3*B*, the kinetics of ERK1/2 activation in response to US was similar to that of ERK phosphorylation, showing a peak activity 20 min after US (1.70 ± 0.23-fold of control).

MEK-1 is an upstream regulator of the ERK cascade. Incubation of cells with a selective MEK-1 inhibitor abolished US-induced ERK1/2 phosphorylation (Fig. 3*C*) and decreased the percentage of BrdU-labeled nuclei in response to US from 19.8 to 8.7% (Fig. 3*D*). This demonstrates that the activation of the ERK cascade is crucial for US-induced DNA synthesis.

ROCK Is an Upstream Mediator of US-induced ERK Activation—Next we examined whether Rho/ROCK and the ERK cascade were independent regulators of US-induced DNA synthesis or parts of a linear signaling cascade. The MEK-1 inhibitor was not able to block US-induced F-actin polymerization and paxillin focal adhesion formation (Fig. 4*A*, *panels c*, *d*, *g*, *h*, *k*, and *l*), suggesting that ERK1/2 activation is not required for US-induced stress fiber and focal adhesion formation. However, treatment of cells with the ROCK inhibitor prevented phosphorylation of ERK1/2 in response to US (Fig. 4*B*), whereas activation of the ERK cascade by transforming growth factor α was not affected by Y27632 in skin fibroblasts (data not shown). Thus, Rho/ROCK is a specific upstream regulator of ERK1/2 activation upon exposure of cells to acoustic pulsed energy.

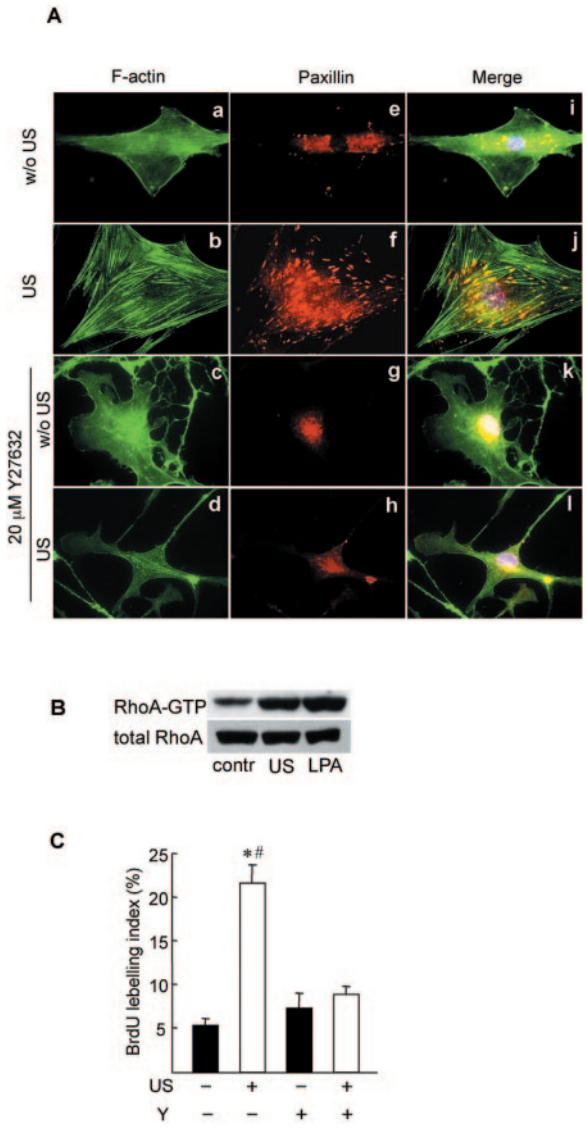


FIG. 2. **US induces polymerization of F-actin and recruitment of paxillin to focal adhesions via activation of Rho kinase, which is required for US-induced BrdU incorporation.** *A*, quiescent fibroblasts grown on glass coverslips were stimulated with US for 11 min in the absence (*panels a*, *b*, *e*, *f*, *i*, and *j*) or presence of 20 μM Y27632 (*panels c*, *d*, *g*, *h*, *k*, and *l*). The cultures were then stopped 45 min after stimulation and fixed with 4% paraformaldehyde in PBS. F-actin filaments were visualized by Alexa-phalloidin (*panels a–d*, *green*); paxillin was determined by indirect immunofluoresence and visualized with Texas Red conjugate (*panels e–h*, *red*); the nuclei were visualized with Hoechst 33258. *Panels i–l* are merged pictures of F-actin, paxillin, and nucleus. *B*, quiescent cultures of skin fibroblasts were stimulated with US for 11 min (lysed 20 min after stimulation) or incubated with 5 μM of lysophosphatic acid (*LPA*) for 2 min or without treatment (*contr*). Rho GTP was detected by using rhotekin RBD affinity precipitation (pull-down assay), as shown in the *upper blot*, and RhoA in total cell lysate as loading control was shown in the *lower blot*. *C*, quiescent cultures of skin fibroblasts were stimulated with US for 11 min in the absence or presence of 20 μM Y27632 (Y). BrdU (50 μM) was added 6 h after US followed by an additional incubation for 18 h. The quantitation of BrdU incorporation was performed as previously described. The data represent the means ± S.E. percentage of BrdU-positive nuclei in three independent wells/condition. *, $p < 0.05$ compared with control cells; #, $p < 0.05$ compared with Y27632 plus US or with Y27632 but without US.

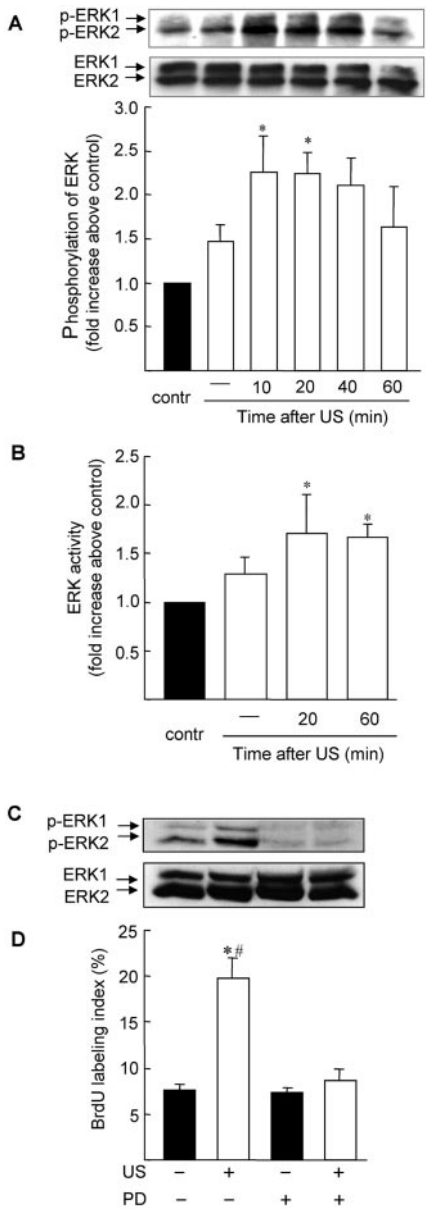
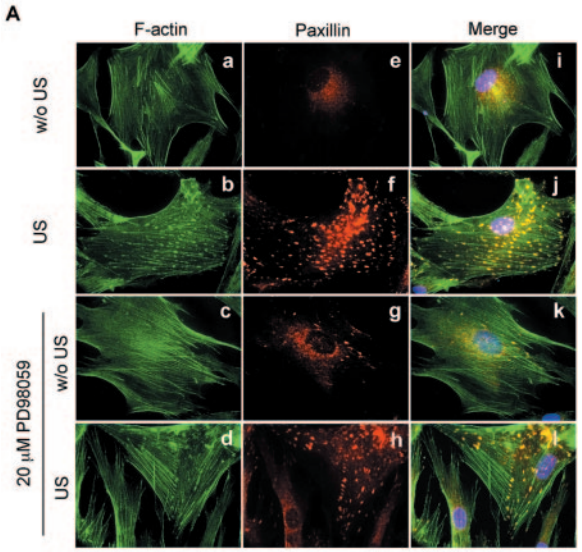


FIG. 3. **US induces activation of ERK1/2, which is required for US-induced BrdU incorporation.** Quiescent skin fibroblasts were stimulated with US for 11 min and lysed at indicated times after stimulation. Phosphorylation of ERK1/2 was analyzed by Western blotting with antibody to threonine and tyrosine dual phosphorylated ERK1/2. Equal protein loading was confirmed by reprobing the stripped blots with antibody to ERK1/2 (*A*, *upper two panels*, and *C*). Quantitation of phosphorylation was performed by scanning densitometry from three separate experiments, presented as the fold increase in phosphorylation of ERK1/2 above control unstimulated level (*A*, *lower panel*). Immune complex kinase assay to examine ERK1/2 activity induced by US was performed as described under "Experimental Procedures." The results are the means of triplicates and expressed as fold increase above control (*w/o US*) (*B*). *, $p < 0.05$ compared with control cells. ERK1/2 phosphorylation and BrdU incorporation in the absence or presence of 20 μM PD98059 are shown as *C* and *D*, respectively. The quantitation of BrdU incorporation was performed as previously described. The data represent the means ± S.E. percentage of BrdU-positive nuclei in three independent wells/condition (*D*). *, $p < 0.05$ compared with control cells; #, $p < 0.05$ compared with PD98059 plus US or with PD98059 but without US.

Effect of Integrin β_1 Blocking Antibody and RGD Peptide on US-induced DNA Synthesis—It has been suggested that integrins serve as mechanotransducers (13, 14). The classical ligand for integrins on fibroblasts is fibronectin, which contains the integrin-recognition sequence RGD (31). Specific integrin blocking antibodies or RGD peptides have been widely used to verify involvement of integrins in mechanotransduction (32–35). To determine whether integrins could mediate the signaling events in response to acoustic pulsed energy, skin fibroblasts were treated with an integrin β_1 blocking antibody



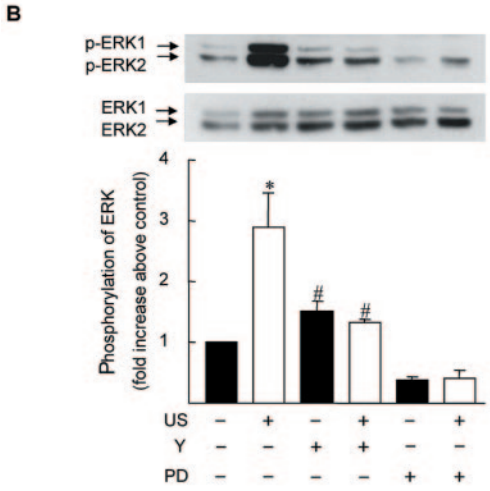


FIG. 4. **Y27632 inhibits US-induced ERK1/2 phosphorylation.** *A*, quiescent fibroblasts grown on glass coverslips were stimulated with US for 11 min in the absence (*panels a*, *b*, *e*, *f*, *i*, and *j*) or presence of 20 μM PD98059 (*panels c*, *d*, *g*, *h*, *k*, and *l*). The cultures were then stopped 45 min after stimulation and fixed with 4% paraformaldehyde in PBS. F-actin filaments were visualized by Alexa-phalloidin (*panels a–d*, *green*); paxillin was determined by indirect immunofluoresence and visualized with Texas Red conjugate (*panels e–h*, *red*); nuclei were visualized with Hoechst 33258. *Panels i*, *j*, *k*, and *l* are merged pictures of F-actin, paxillin, and nucleus. *B*, quiescent skin fibroblasts were incubated with 20 μM Y27632 (*Y*) or 20 μM PD98059 (*PD*) for 1 h followed by 11 min of US stimulation. The cells were lysed 20 min after US stimulation. Phosphorylation of ERK1/2 was analyzed by Western blotting as described previously (*A*, *upper two panels*). Quantitation of phosphorylation was performed by scanning densitometry from three separate experiments, presented as the fold increase in phosphorylation of ERK1/2 above control unstimulated level (*A*, *lower panel*). *, $p < 0.05$ compared with control cells.

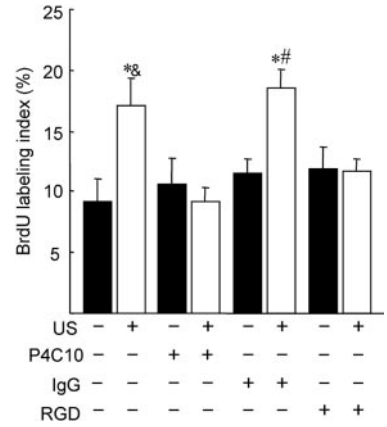


FIG. 5. **Integrin β_1 blocking antibody and RGD peptide attenuate US-induced BrdU incorporation.** Quiescent subconfluent skin fibroblasts were preincubated with 10 μg/ml integrin β_1 blocking antibody (P4C10), 100 μg/ml RGD peptide (H-Arg-Gly-Asp-OH), or 10 μg/ml mouse IgG (IgG) for 3 h. The cells were then stimulated with US for 11 min, and BrdU incorporation was determined as previously described. The data represent the means ± S.E. percentage of BrdU-positive nuclei in three independent wells per condition. *, $p < 0.05$ compared with control cells; &, $p < 0.05$ compared with integrin β_1 blocking antibody or RGD peptide; #, $p < 0.05$ compared with cells with mouse IgG but without US.

(P4C10) as well as a RGD peptide. Both treatments reduced US-induced BrdU incorporation to base-line levels (Fig. 5). In striking contrast, pretreatment of the cells with an unspecific mouse IgG as a control had no inhibitory effect on US-induced BrdU incorporation (Fig. 5). Thus, integrins are mediators of acoustic pulsed energy-induced BrdU incorporation in primary skin fibroblasts.

US-induced ERK Activation Is Independent of EGFR Tyrosine Kinase Activity in Primary Skin Fibroblasts—The data above demonstrate that integrins mediate US-induced DNA synthesis in skin fibroblasts by an ERK-dependent mechanism. It has been demonstrated that integrin-dependent adhesion can trigger ligand-independent EGFR activation to transduce downstream signaling (25). Therefore we examined whether US-induced ERK activation required EGFR activity. As shown in Fig. 6*A*, the selective EGFR tyrosine kinase inhibitor AG1478 did not prevent ERK1/2 phosphorylation in response to acoustic pulsed energy. However, the inhibitor abolished ERK1/2 phosphorylation and EGFR phosphorylation in response to transforming growth factor α, a ligand of the EGFR. In accordance with these data, we could not detect an increase in tyrosine phosphorylation of the EGFR in response to US (Fig. 6*B*). Thus, intracellular signaling induced by acoustic pulsed energy is independent of EGFR signaling and does not induce EGFR transactivation.

Src Is an Upstream Regulator of US-induced ERK Activation, Whereas PI 3-Kinase/Akt Pathway Is Not Involved in US-triggered Signaling—The tyrosine kinase Src has also been implicated in the activation of the ERK cascade by integrins (36). Indeed, US induced a slight increase in phosphorylation of Src at Tyr^{416} in the activation loop with similar kinetics as ERK1/2 phosphorylation (Fig. 7*A*). The selective Src inhibitor PP1 blocked US-induced Src activation. In addition, PP1 also inhibited ERK1/2 phosphorylation, indicating that Src is an upstream regulator of US-induced ERK activation in primary human skin fibroblasts (Fig. 7*B*). β_1 integrins have also been demonstrated to activate the PI 3-kinase/AKT signaling pathway, which is implicated in the regulation of apoptosis and cell cycle progression (37). However, we detected neither an inhibition of US-induced DNA synthesis by the selective PI 3-kinase inhibitor LY294002 nor an increase in phosphorylation of the PI 3-kinase downstream target Akt in response to US stimulation (data not shown). Thus, the PI 3-kinase/Akt pathway is not involved in the signaling cascade induced by acoustic pulsed energy in primary skin fibroblasts.

DISCUSSION

Tissue repair is a complex process involving different cell types and biochemical reactions. It is usually divided into three overlapping phases, the inflammatory phase, the proliferative phase, and the remodeling phase (38). Acoustic pulsed energy

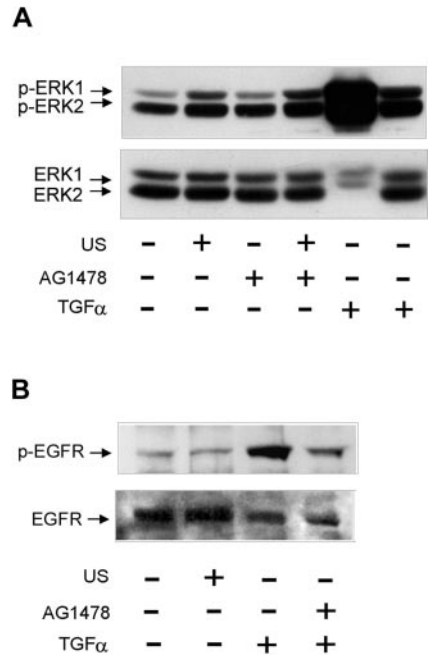


FIG. 6. **Acoustic pulsed US does not transactivate EGFR.** Quiescent confluent skin fibroblasts were preincubated with 0.2 μM of AG1478 for 1 h and treated with US for 11 min or 50 ng/ml of transforming growth factor α for 10 min. The total cell lysates were obtained at 11 min after US stimulation. Phosphorylation of EGFR and ERK1/2 was analyzed by Western blotting by using anti-phospho-ERK1/2 and anti-phospho-EGFR (Tyr^{1068}) antibodies. Equal protein loading was confirmed by reprobing the stripped blots with antibody to ERK1/2 and EGFR. The blots are representative of three independent experiments.

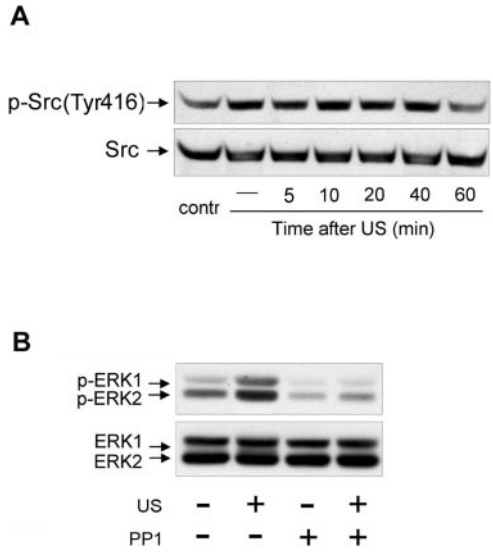


FIG. 7. **Src acts as an upstream regulator of US-induced ERK1/2 activation.** *A*, quiescent confluent skin fibroblasts were stimulated with US for 11 min and lysed at indicated times after stimulation. *B*, cells were preincubated with a Src family inhibitor PP1 (5 μM) for 1 h and stimulated with US for 11 min, and total cellular lysate was obtained 20 min after US stimulation. Phosphorylation of ERK1/2 and Src (Tyr^{416}) were examined by Western blotting using phospho-specific antibodies. Total ERK and Src were reprobed after stripping as loading control. The blots are representative of three independent experiments.

is increasingly used as a supplementary therapy to promote bone and wound healing (5), but the underlying molecular mechanisms are poorly understood. Fibroblasts are the major cell type in connective tissue and are critical for tissue restoration and remodeling after injury. Therefore, primary skin fibroblasts are a useful model to understand the mechanism of US to promote tissue repair. In the present report, we show that a single low intensity pulsed US treatment is able to promote DNA synthesis in quiescent primary skin fibroblasts. Daily repeated US stimulation, which resembles the clinical schedule, substantially increases cell numbers. These experiments demonstrate that a physical stimulus, acoustic pulsed energy, can promote cell proliferation, which is a prerequisite of wound healing.

Mechanical stimuli can affect both the actin cytoskeleton and cell cycle progression (39). The Rho family of small GTPases and downstream targets such as ROCK regulate cytoskeletal organization (40), in particular the formation of stress fiber and focal adhesion complexes (19, 41). Our data show that US activates RhoA, induces the formation of F-actin stress fibers, and recruits paxillin to focal adhesions by a ROCK-dependent pathway. In addition, the activation of Rho/ROCK is required for US-induced BrdU incorporation.

Direct mechanical stimulation such as stretching of cells can activate the ERK cascade in different cell systems (2, 4, 13, 32, 33, 41), leading to cell proliferation (42). Acoustic pulsed energy can be regarded as a form of nonphysiological mechanical energy applying mechanical stress indirectly to the cultured cells. US induced an approximately 2-fold transient increase in ERK1/2 activation in primary human foreskin fibroblasts, which is comparable with direct mechanical stimulation (2, 32, 33). Furthermore activation of ERK1/2 by US was required to trigger DNA synthesis in primary human skin fibroblasts.

It has been suggested that Rho could be involved in integrin-induced activation of ERK2 in fibroblasts (43). Our data further establish that RhoA/ROCK acts as an upstream regulator of the ERK cascade to induce cell proliferation in response to US stimulation in primary human skin fibroblasts. Integrins act as mechanotransducers in response to shear stress (32), mechanical stretch (33), and cycled magnetic fields (4). Our results show that β_1 integrins mediate US-induced BrdU incorporation, indicating that integrin receptors on skin fibroblasts serve as mechanotransducers for acoustic pulsed energy. It has become apparent that the EGFR participates in signaling networks activated by multiple extracellular stimuli such as adhesion, lymphokines, or stress signals in the absence of EGFR ligands (44). Direct mechanical stimulation of cells by cyclic stretching induces ERK1/2 activation via transactivation of the EGFR (45, 46). Our data show that acoustic pulsed energy, which employs integrins as mechanotransducer in skin fibroblasts, activates ERK pathway without participation of EGFR activity. The tyrosine kinase Src and PI 3-kinase/Akt have been shown to play a role in integrin-stimulated signaling (36, 37). Our data demonstrate that acoustic pulsed energy triggers Src activation, which in turn regulates the activation of the ERK signaling pathway in primary skin fibroblasts. In contrast, PI 3-kinase/Akt is not involved in US-induced intracellular signaling.

In conclusion, this report establishes that acoustic pulsed energy induces proliferation of primary skin fibroblasts and provides a molecular basis for the clinical observation that ultrasound treatment of wounds can promote tissue repair. In addition, we delineate the complex network of signaling pathways that is triggered by acoustic pulsed energy in primary fibroblasts, demonstrating that US-induced proliferation involves the activation of β_1 integrins and RhoA/ROCK- and Src-ERK signaling cascade. However, integrin activation and cell proliferation in response to acoustic pulsed energy does not involve transactivation of the EGFR, demonstrating that this type of mechanical stimulus triggers a specific signaling platform.

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