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Superoxide Mediates Shock Wave Induction of ERK-dependent Osteogenic Transcription Factor (CBFA1) and Mesenchymal Cell Differentiation toward Osteoprogenitors*

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Extracorporeal shock wave (ESW) is an alternative non-invasive method for the promotion of bone growth and tendon repair. In an animal model, we have reported that ESW promoted bone marrow osteoprogenitor growth through transforming growth factor-β1 induction. We have further explored the mechanism for the ESW promotion of osteogenesis. Results showed that an optimal ESW treatment at 0.16 mJ/mm^2 for 500 impulses rapidly induced a higher O_2^- and $ONOO^-$ production associated with a decrease of nitric oxide level in 1 h, and induced a higher transforming growth factor-β1 production in 24 h, and a higher colony-forming units-osteoprogenitor formation in 12 days. The colony-forming units-osteoprogenitor colonies revealed positive staining of bone alkaline phosphatase and turned into bone nodules in 21 days. Early scavenging of O_2^- but not Ca^{2+}, H_2O_2, or prostaglandin E_2 suppressed osteoprogenitor cell growth and maturation. Scavenging of O_2^- by superoxide dismutase raised the nitric oxide level back to the basal level and suppressed ESW-promoted osteoprogenitor cell growth, whereas inhibition of $ONOO^-$ by urate or NO by *N*-nitro-L-arginine methyl ester did not affect ESW promotion of osteogenesis, indicating that O_2^- acted as an early signal for ESW-induced cell growth. Further studies demonstrated that ESW induced ERK activation, and blockage of O_2^- production or inhibition of tyrosine kinase, but not protein kinase A and C inhibitors, suppressed ESW-induced ERK activation. In support that O_2^- mediated the ESW-induced ERK activation and osteogenic differentiation, we further demonstrated that scavenging of O_2^- by superoxide dismutase and inhibition of ERK activation by PD98059 decreased specific osteogenic transcription factor, core binding factor A1 activation, and decreased osteocalcin expression. Taken together, we showed that ESW-induced O_2^- production followed by tyrosine kinase-mediated ERK activation and core binding factor A1 activation resulted in osteogenic cell growth and maturation. Thus, an appropriate modulation of redox reaction by ESW may have some positive effect on the bone regeneration.

Oxidative stress induced by superoxide has been implicated in the induction of certain cell injury (1–5). In contrast, superoxide also plays an important role in the regulation of cell proliferation and metabolism (6–8). Several physical factors such as heat (9), electrical field (10), pulsatile stretch (11), and laser irradiation (12) can stimulate cell proliferation through the involvement of superoxide. It is not known whether superoxide can regulate osteoprogenitor cell growth and differentiation.

Extracorporeal shock wave (ESW)[1] is created by a high voltage spark discharge under water causing an explosive evaporation of water and producing high energy acoustic waves. The acoustic waves are focused on a semi-ellipsoid reflector and therefore can be transmitted into a specific tissue site (13). ESW treatment has been divergently applied for eukaryotic and prokaryotic biology systems. It is well known that ESW provides a non-invasive biophysical strategy for breaking renal stones with minimal side effects (13). Evidence also suggests that shock waves can potentially enhance gene transfer (14), suppress tumor growth (15), and promote the bactericidal effect of microorganisms (16).

Recently, we and others (17–20) have shown that ESW treatment has a promising effect on the promotion of bone fracture healing and repair of tendinitis. The mechanism by which ESW enhances fracture healing and repair of tendinitis remains to be determined. The fact that ESW treatment enhances both bone and tendon regeneration suggests that ESW may induce a certain signal for growth and maturation of the mesenchymal progenitors from bone marrow. It has been well clarified that the differentiation and maturation of bone marrow mesenchymal osteoprogenitor cells into osteoblastic lineage is involved in bone regeneration (21–23). In support of the hypothesis, we have recently shown ESW treatment to be able to promote bone marrow stromal cell growth and differentiation toward osteogenic lineage, presumably through TGF-β1 induction (24).

Accumulated evidence suggests that ESW induces a cavitation effect to increase membrane permeability and the influx of biological substances (25, 26), which are usually implicated in cell and tissue damage (15, 27). There is limited evidence showing that ESW promotes cell growth rather than cell damage. We hypothesized in this study that an optimal ESW treatment promoted osteoprogenitor cell growth and maturation via a rapid induction of oxygen radicals for a signal transduction

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[1] The abbreviations used are: ESW, extracorporeal shock wave; TGF-β1, transforming growth factor-β1; CFU-O, colony-forming unit-osteoprogenitor; ERK, extracellular signal-regulated kinase; CBFA1, core binding factor A1; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SOD, superoxide dismutase; L-NAME, *N*-nitro-L-arginine methyl ester; PKA, protein kinase A; PKC, protein kinase C; MBP, myelin basic protein; PGE_2, prostaglandin E_2; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline.

from ERK to specific osteogenic transcription factor activation, followed by osteogenesis. Thus, we have sought to investigate which species of oxygen radicals could be induced by ESW treatment, how early the oxygen radicals transmitted the signal cascade, when the growth factor TGF-β1 was induced, and when the growth and osteogenic maturation was reached.

EXPERIMENTAL PROCEDURES

ESW Treatment of Bone Marrow Cells ex Vivo—Three-month-old Sprague-Dawley rats (National Experimental Animals Production Center, Taipei, Taiwan) were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg; Nembutal® sodium, Abbott). Each rat was placed in supine position with four limbs abducent fixation. The ESW treatment with 0, 250, 500, and 1000 impulses at 0.16 mJ/mm^2 (Ossatron®; HMT High Medical Technologies GmbH, Kreuzlingen, Switzerland) was applied to the left distal femur 10 mm above the knee joint as in our previous study (24). After ESW treatment for 1 h, bone marrow cells were harvested from the bone marrow of femurs with ESW treatment. Bone marrow cells from the femurs without ESW treatment were run as controls.

Preparation of Rat Bone Marrow Mononuclear Cells—The distal end of the femur bone was excised at the level of 5 mm above the knee joint. Bone marrow blood (0.4 ml) was aspirated with 20-gauge needle into a 1-ml syringe containing 20 units/ml heparin. The bone marrow mononuclear cells in the marrow blood were harvested from the interface of the Ficoll-Paque density gradient (d = 1.007 g/ml, Amersham Biosciences AB) at 500 × g for 30 min as described previously (28). Cell number and viability were determined with a hemocytometer after staining with 0.4% trypan blue in ammonium chloride.

Culture of Bone Marrow Osteoprogenitor Cells—Culture of the osteoprogenitors from bone marrow mononuclear cells (2 × 10^5 cells/well; 24-well plate) was raised in osteogenic medium containing Dulbecco's modified Eagle medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS), 10^{-8} M dexamethasone, 50 μg/ml L-ascorbic acid, and 10^{-2} M β-glycerophosphate (Sigma). After washing out nonadherent hematopoietic cells, total stromal cells were cultured for 12 days at 5% CO_2 and incubated at 37 °C. The cultured supernatant was harvested and replaced with fresh osteogenic medium every 3 days. Colony-forming unit-osteoprogenitor (CFU-O) in bone marrow stromal cell culture was assessed as described previously (29). After incubation and removal of medium, the cell culture in each well was fixed with citrate/acetone/formaldehyde and subjected to bone alkaline phosphatase staining according to the instructions for the use of Sigma alkaline phosphatase histocytochemistry assay kit (Sigma). Total colonies found to have more than 32 segregate cells were recognized and calculated as positive bone alkaline phosphatase staining.

Determination of Alkaline Phosphatase Activity—The cells (1 × 10^4 cells/well; 96-well plate) from CFU-O colonies were subjected to determination of the osteoblastic lineage with bone alkaline phosphatase activity (30). The reactions were incubated with 0.2 ml of substrate buffer containing 50 mM glycine, 1 mM magnesium chloride, pH 10.5, and 2.5 mM p-nitrophenyl phosphate (Sigma) at 37 °C for 30 min and stopped with 0.1 ml of 1 N sodium hydroxide. Results were read at $A_{405\ nm}$ by a microplate reader (Dyn-Ex Technologies Inc.). Alkaline phosphatase activity was expressed as μM p-nitrophenol/well (31).

Measurement of Bone Nodule Formation—To confirm further the osteogenic formation, we also prolonged the primary osteoprogenitor cell culture to 21 days. The long term cultured CFU-O colonies were fixed with neutral buffered formaldehyde for 5 min, pH 7.4, rinsed with distilled water, and then stained using the von Kossa method with 0.3 ml of 5% freshly prepared silver nitrate. After this procedure, the size of bone nodules ≥2 mm^2 showing positive von Kossa staining were counted under an inverted microscope as described previously (32).

ESW Treatment of Bone Marrow Stromal Cell in Vitro—Rat bone marrow stromal cells harvested from rat femurs were cultured in DMEM with 10% FBS. After washing out nonadherent hematopoietic cells, total stromal cells were cultured for 5 days in a 5% CO_2, 37 °C incubator into log phase growth before harvest. To study the role of Ca^{2-} in ESW-promoted cell growth, cells (1 × 10^6 cells/ml) were treated with the extracellular Ca^{2+} chelator, 1 mM EGTA, or the intracellular Ca^{2+} chelator, 40 μM BAPTA (Sigma), for 30 min. To study the role of superoxide or hydrogen peroxide in the ESW-promoted cell growth, cells were treated for 30 min with 500 units/ml SOD or catalase (Sigma) to scavenge superoxide and hydrogen peroxide (33). To elucidate whether prostaglandin E_2 was involved in the ESW-enhanced cell growth, cells were treated with 10 μM indomethacin to suppress PGE_2 production by inhibiting cyclooxygenase-2 activity (34). In studies of the ESW-induced signal transduction of cell growth, cells were treated with 20 μM PD98059, a MEK inhibitor (Calbiochem), 50 μM calphostain C, a protein kinase A inhibitor, 100 μM (R_p)-cAMP, a protein kinase C inhibitor, and 20 μM genistein (Sigma), a tyrosine kinase inhibitor (35), for 60 min before studies. After these treatments, cells were washed and resuspended with PBS. Cell suspensions were subjected to ESW treatment with modification as described previously (36). Briefly, cells (1 × 10^6 cells/ml) were suspended in a 5-ml polystyrene round-bottom tube (Falcon®, Becton Dickinson) containing 5 ml of PBS at pH 7.4 and exposed to ESW at 0.16 mJ/mm^2 for 500 impulses. The duration of ESW treatment took 10 min. After ESW treatment, cells were cultured for 1 h and 2 days as indicated in the cell proliferation and Western blot assays. In some experiments, cells (1 × 10^5 cell/well, 24-well plate) were cultured in osteogenic medium for 12 and 21 days in order to elucidate the osteoprogenitor maturation as demonstrated by osteocalcin expression and bone nodule formation. Cultured medium was harvested and replaced with fresh medium every 3 days. The long term cell cultures in each well were subjected to Western blot assay of osteocalcin production and von Kossa staining of bone nodule formations.

Determination of Superoxide Production by Bone Marrow Stromal Cells—To detect kinetic change of superoxide production, ESW-treated cells (1 × 10^5 cells/well, 96-well plate) were incubated with phenol red-free DMEM for 1, 6, 24, and 48 h. After incubation, each well was added with 50 μM horse heart cytochrome c (Sigma) and followed by incubation in the dark at 37 °C for 1 h. The O_2^- production was determined by the difference of cytochrome c reduction in the absence and presence of SOD. The cytochrome c reduction was monitored at $A_{550\ nm}$, and O_2^- concentration was calculated from the molar extinction coefficient of 0.0282 μM^{-1} cm^{-1} to calculate O_2^- production (33). To elucidate whether O_2^- was an early or late signal for ESW promotion of bone marrow stromal cell growth, cells in each well were added with 500 units/ml SOD or vehicle (PBS) for 30 min at 0, 6, and 24 h after ESW treatment and subjected to O_2^- production assay. To elucidate whether O_2^- was an early or late signal for ESW promotion of TGF-β1 induction and bone marrow stromal cell growth, cells in each well were treated with 500 units/ml SOD or vehicle for 30 min at 0, 6, and 24 h after ESW treatment. The supernatant of the reactions was harvested for TGF-β1 production in 1 day, and the cells were subjected to the cell proliferation assay in 2 days. The changes of O_2^- production were correlated to the stromal cell growth, TGF-β1 production, and bone nodule formations as well as signal transduction as described below.

Measurement of NO Production by Bone Marrow Cells—To elucidate the role of NO or $ONOO^-$ in the ESW-promoted cell growth, cells were treated for 30 min with and without 100 μM urate or 100 μM L-NAME (Sigma) to inhibit $ONOO^-$ or NO production (37, 38). After washing and resuspension in PBS, cells were subjected to ESW treatment. ESW-treated cells (1 × 10^5 cells/well, 96-well plate) were incubated with phenol red-free DMEM for 1 h. Cultured supernatant was harvested for assessment of NO production. Cells were collected for determination of nitrotyrosine, a marker for $ONOO^-$ formation. In some experiments, the supernatants of the reactions were harvested for TGF-β1 production in 1 day, and the cells were subjected to von Kossa assay in 21 days. We measured nitrite and nitrate levels to reflect NO production as described previously (39). Briefly, the culture supernatants were determined in triplicate by injecting the 50-μl aliquot into a custom impinger with a Teflon-linked septum containing 0.4 M vanadium chloride in glacial acetic acid. The vanadium chloride reduced nitrate and nitrite to NO gas, which passed into a stream of helium and entered the nitrogen oxide analyzer (NOA280; Sievers Inc., Denver, CO) as described previously (39). The nitrite and nitrate levels in each sample were determined by interpolation calculated from a series of well known potassium nitrate concentrations. Protein concentration in each sample was measured by a Bio-Rad protein assay kit (Bio-Rad). Results were normalized with protein concentration in each sample.

Measurement of Peroxynitrite as Determined by Nitrotyrosine—To investigate the interaction of O_2^- and NO, we measured the nitrotyrosine level, which is a marker for $ONOO^-$ formation, as described previously (40). The nitrotyrosine levels in each sample were determined by high performance liquid chromatography (LC-10AD, Shimadze, Tokyo, Japan) equipped with a reverse phase column (4.6 mm internal diameter × 250 mm length; TSK-gel, ODS-80TM). The column was eluted with 50 mM NaH_2PO_4·H_3PO_4 (pH 2.5) with methanol (60:40, v/v) at a flow rate of 2 ml/min through an isocratic pump. The peaks were measured with an electrochemical detector (Coulochem II, Bedford, MA) at a guard cell potential of −250 mV. The nitrotyrosine concentration in each sample was integrated from the retention time and area under the eluting peak. The exact concentration was determined by an interpolation calculated from a series of well known stand-

ard concentrations of 3-nitrotyrosine (Calbiochem). Results were normalized with protein concentration in each sample (40).

Measurement of TGF-β1 Production in the Culture Supernatants—The cultured supernatants were harvested for measurement of TGF-β1 by centrifuging at 500 × *g* for 5 min and then stored at −70 °C until studied. The TGF-β1 production was determined by an enzyme-linked immunosorbent assay (Quantikine®, R & D Systems Inc.). Briefly, acid-activated culture supernatants (0.2 ml) were added to each polystyrene microwell pre-coated with recombinant human TGF-β-soluble receptor type II for 3 h. The reactions were next incubated with a horseradish peroxidase-conjugated TGF-β1 polyclonal antibody for 1.5 h. After washing, the reactions were incubated with substrate solution containing 0.1 ml of stabilized hydrogen peroxide and 0.1 ml of stabilized tetramethylbenzidine for 30 min. The reaction was stopped with 0.05 ml of 2 N sulfuric acid. Data were read at $A_{450\ nm}$ with a microplate reader. Results were calculated by an interpolation from a standard curve made by a series of TGF-β1 concentrations.

Proliferation of Bone Marrow Stromal Cells—Bone marrow stromal cell proliferation was determined by nuclear [^{3}H]thymidine uptake as modified from a measurement of osteoblast proliferation described previously (36). Bone marrow stromal cells with and without ESW treatment (2×10^4 cells/well, 96-well plate) were cultured for 24 h before the addition of 1 μCi of [^{3}H]thymidine/well (Amersham Biosciences AB) for an additional 24-h culture. At the end of the culture period, cells in each culture well were released from the plates by trypsinization and processed for [^{3}H]thymidine uptake determination by a liquid scintillation analyzer (Tri-Crab 2100TR, Packard Instrument Co.).

Preparation of Cytosolic and Nuclear Extract—Bone marrow stromal cells were lysed with 200 μl of ice-cold buffer containing 10 mM Tris, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.7% Nonidet P-40 on ice for 10 min and centrifuged at 500 × *g* for 5 min. The cytosolic extracts were harvested to measure ERK activation. The nuclear pellets were further lysed with buffer containing 40 mM Tris, pH 7.9, 350 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM dithiothreitol, 2 μg/ml leupeptin, and 1 μg/ml aprotinin on ice for 20 min and harvested to determine osteogenic transcription factor and core binding factor A1 (CBFA1) activation by centrifugation at 12,000 × *g*, 4 °C, for 10 min. Protein concentrations in cytosolic and nuclear extracts were determined by Bio-Rad assay kit (Bio-Rad).

Measurement of ERK Activation—The cytosolic extracts were incubated with anti-ERK antibody (1:100; Upstate Biotechnology, Inc.) for 1 h at 4 °C. After incubation, the immune complexes were precipitated with protein A (Sigma). The immunoprecipitate (20 μg) was reacted with the substrate buffer containing 20 μg of myelin basic protein (MBP), 15 mM $MgCl_2$, 100 μM ATP, and 5 μM protein kinase inhibitor for 30 min at 30 °C. The reaction was stopped with the Laemmli buffer containing 200 mM Tris, pH 6.8, 10% glycerol, 4% SDS, 50 μM dithiothreitol, and 0.05% bromphenol blue for 5 min at 95 °C. The mixtures were subject to Western blot assay. The phosphorylated MBP on the blot was recognized by a specific mouse anti-phospho-MBP (1:1000) antibody, followed by goat anti-mouse horseradish peroxidase-conjugated IgG (1:3000) as the second antibody. The ERK activity was reflected on the phosphorylated MBP visualized with chemiluminescence agents.

Measurement of CBFA1 Phosphorylation—The nuclear extracts were incubated with anti-CBFA1 antibody (1:100; Santa Cruz Biotechnology) for 1 h at 4 °C. After incubation, the immune complexes were precipitated with protein A (Sigma). The immunoprecipitate (20 μg) was mixed with Laemmli buffer for 5 min at 95 °C. The mixtures were subject to Western blot assay. The total CBFA1 on the blot was recognized by a rabbit anti-CBFA1 antibody at 1:500 dilution, followed by goat anti-rabbit horseradish peroxidase-conjugated IgG (1:2000) as the second antibody. The phosphorylated CBFA1 on the blot was further recognized by a specific mouse anti-phosphotyrosine (1:1000) antibody, followed by goat anti-mouse horseradish peroxidase-conjugated IgG (1:2000) as the second antibody. The CBFA1 activity was reflected on the phosphorylated CBFA1 visualized with chemiluminescence agents.

Measurement of Osteocalcin Production—To confirm ESW promotion of osteoprogenitor maturation, cytosolic extracts were subjected to determination of osteocalcin production with immunoblot assay. The osteocalcin on the blot was recognized by a specific mouse anti-osteocalcin (1:500) antibody, followed by goat anti-mouse horseradish peroxidase-conjugated IgG (1:3000) as the second antibody. The osteogenic activity was reflected on the osteocalcin expression visualized with chemiluminescence agents.

Statistics Analysis—Data were analyzed with a non-parametric one-way analysis of variance followed by Student's *t* test to determine significance between treatments. $p < 0.05$ was considered statistically significant.

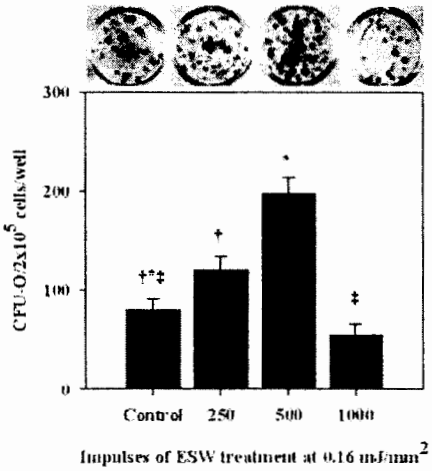

FIG. 1. **Effects of various doses of ESW treatment on the CFU-O formation.** The left femurs of rats were treated with and without 0.16 mJ/mm^2 ESW for 250–1000 impulses. Bone marrow stromal cells (2×10^5 cells/well, 24-well plate) were harvested and cultured in DMEM with 10% FBS, 10^{-9} M dexamethasone, 10^{-2} M β-glycerophosphate, and 50 μg/ml ascorbic acid for 12 days. CFU-O formations were determined by colonies with ≥32 segregated cells showing positive bone alkaline phosphatase staining. † ($p = 0.037$), * ($p < 0.001$), and ‡ ($p = 0.028$) represent a significant difference between both groups. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

RESULTS

Optimal Dose of ESW Treatment Promoted CFU-O Growth and Bone Nodule Formation—Certain doses of ESW treatments applied to rat femoral bone promoted the CFU-O formation of bone marrow stromal cells. As shown in Fig. 1, the ESW treatment with 0.16 mJ/mm^2 for 250 impulses minimally enhanced CFU-O formation, and the treatment for 500 impulses had the best effect. However, the treatment with 1000 impulses brought about a suppressing effect. The CFU-O colonies were confirmed to be osteoblastic lineage, as demonstrated by an increase in alkaline phosphatase activity in the cells from CFU-O colonies (Fig. 2*A*). The cells from the CFU-O colonies matured into bone nodules after a 21-day long term culture as shown in Fig. 2*B*. The bone nodule formations were significantly higher in the ESW treatment with 500 impulses than those without ESW treatment (Fig. 2*B*).

ESW Promotion of Osteoprogenitor Cell Growth Mediated by O_2^- but Not Ca^{2+}, H_2O_2, or PGE_2—ESW is a high energy acoustic wave and is postulated to induce cell or tissue damage by alteration of calcium homeostasis (41) or reactive oxygen radicals (42) as described previously. Evidence has demonstrated that induction of PGE_2 is involved in ultrasound stimulation of mouse osteoblast growth (43). We sought to elucidate whether a certain mediator was involved in the ESW promotion of osteoprogenitor cell growth. It was found that chelation of extracellular Ca^{2+} with 1 mM EGTA or chelation of intracellular Ca^{2+} with 40 μM BAPTA did not affect the ESW promotion of osteoprogenitor cell growth as demonstrated by [^{3}H]thymidine incorporation (Fig. 3). Scavenging of hydrogen peroxide by 500 units/ml catalase did not influence ESW-promoted cell growth. Inhibition of COX-2 activity by 10 μM indomethacin, which was a critical enzyme for PGE_2 production, did not affect ESW-promoted cell growth (Fig. 3). Nevertheless, the addition of SOD (500 units/ml) significantly ($p = 0.019$) suppressed ESW enhancement of osteoprogenitor proliferation (Fig. 3). Results from these studies suggest that O_2^-, but not Ca^{2+},

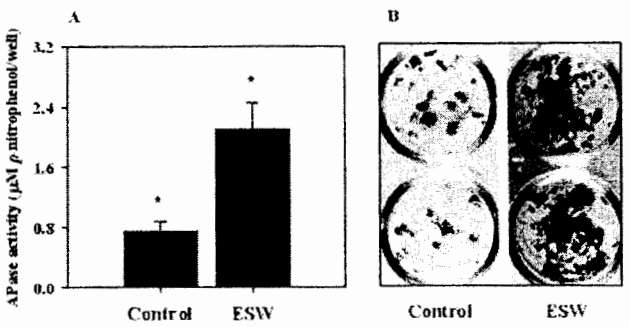

FIG. 2. **ESW promoted bone growth as determined by alkaline phosphatase activity and bone nodule formations.** *A*, ESW promotion of bone alkaline phosphatase activities. Cells (1×10^4 cells/well, 96-well plate) sub-cultured from the CFU-O colonies with and without ESW treatment at 0.16 mJ/mm^2 for 500 impulses were subjected to assessment of alkaline phosphatase activity. * indicates a significant difference between both groups ($p < 0.001$). *B*, ESW promotion of bone nodule formations as determined by von Kossa staining. Bone marrow stromal cells (2×10^5 cells/well, 24-well plate) with and without ESW treatment for 500 impulses were cultured for 21 days before von Kossa staining. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

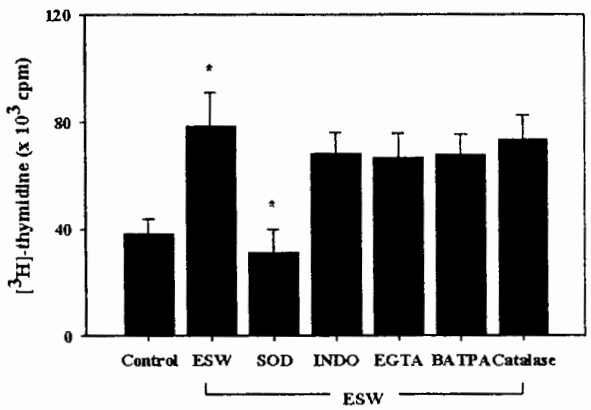

FIG. 3. **Superoxide, but not Ca^{2+}, H_2O_2, or PGE_2, was involved in ESW promotion of bone marrow stromal cell growth.** Cells (1×10^6 cells/ml) were treated with extracellular Ca^{2+}-chelating agent (1 mM EGTA) or intracellular Ca^{2+}-chelating agent (10 mM BAPTA), 500 units/ml superoxide dismutase (500 units/ml), catalase (500 units/ml), or indomethacin (*INDO*; 10 μM) for 30 min and exposed to ESW treatment at 0.16 mJ/mm^2 for 500 impulses. Cell growth was determined by [^{3}H]thymidine incorporation. * indicates a significant difference between both groups ($p = 0.019$). Results are presented by mean values ± S.E. calculated from six duplicate experiments.

H_2O_2, or PGE_2, was involved in the ESW promotion of osteoprogenitor cell growth.

ESW Treatment Induced Superoxide Production followed by TGF-β1 Production—Experiments were next performed to investigate biological responses of bone marrow stromal cells after ESW treatment. We sought to investigate whether the acoustic ESW could raise superoxide production contributing to the induction of osteogenic growth factor such as TGF-β1. It was found that the ESW treatment at 500 impulses significantly increased ($p < 0.001$) the O_2^- production in 1 h (Fig. 4*A*). This higher production of O_2^- persisted for 24 h. To explore whether the O_2^- production was associated with the osteogenic growth factor, TGF-β1 induction, we demonstrated that TGF-β1 production was not immediately induced until 24 h after ESW treatment (Fig. 4*B*).

ESW-promoted Osteogenesis Mediated by O_2^- but Not $ONOO^-$—It is well known that O_2^- can react rapidly with NO to generate $ONOO^-$ (37), which may mediate another signal

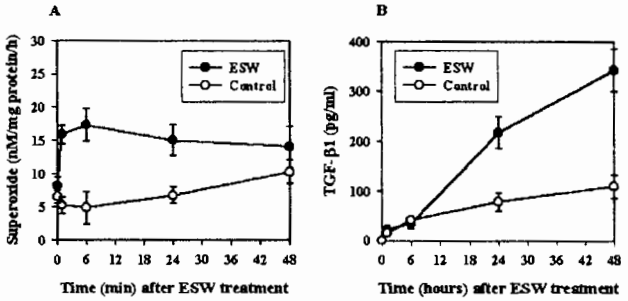

FIG. 4. **ESW induction of superoxide and TGF-β1 production by bone marrow stromal cells.** Bone marrow stromal cells (1×10^6 cells) from the femurs with and without 0.16 mJ/mm^2 ESW treatment for 500 impulses were subjected to assessment of superoxide (*A*) and TGF-β1 (*B*) production at 0, 1, 6, and 24 h. Results are presented by mean values ± S.E. calculated from six duplicate experiments.

for cell growth or damage. We added 500 units/ml SOD, 100 μM urate, or L-NAME to the cell culture right before the ESW treatment to determine whether O_2^-, NO, or $ONOO^-$ was involved in ESW promotion of osteogenesis. As shown in Table I, ESW treatment induced a rapid increase of O_2^- and nitrotyrosine production associated with a decrease in NO production. Addition of SOD significantly decreased ESW-induced O_2^- and nitrotyrosine production but raised the NO level back to the control level (Table I). The addition of urate (100 μM) to scavenge $ONOO^-$ did not affect ESW-augmented O_2^- or NO levels but significantly suppressed nitrotyrosine production. Moreover, scavenging of $ONOO^-$ did not affect ESW-promoted TGF-β1 production or bone nodule formations (Table I). However, addition of 100 μm L-NAME significantly decreased NO and $ONOO^-$ levels but did not affect ESW-enhanced O_2^- production, TGF-β1 production, or bone nodule formation (Table I).

Early but Not Late Scavenge of O_2^- by SOD-suppressed TGF-β1 Production, Osteoprogenitor Growth, and Bone Nodule Formations—We added SOD (500 units/ml) at 0, 6, and 24 h after ESW treatment to determine whether ESW-induced O_2^- acted an early or late signal for bone marrow mesenchymal cell growth. As shown in Fig. 5*A*, the addition of SOD at 0, 6, or 24 h after ESW treatment effectively scavenged ESW-induced O_2^- production. We also found that the early addition of SOD right after ESW treatment inhibited TGF-β1 production by bone marrow stromal cells in 24 h, whereas the addition of SOD at 6 h or 24 h after ESW treatment, although suppressing O_2^- production, did not significantly suppress TGF-β1 production (Fig. 5*B*). Similarly, the early addition of SOD at 0 h, but not at 6 or 24 h, suppressed ESW-promoted cell growth as demonstrated by [^{3}H]thymidine incorporation ($p < 0.001$) (Fig. 5*C*). Moreover, it was found that the early addition of SOD did significantly suppress the ESW-augmented bone nodule formations (Fig. 5*D*).

ESW-promoted Cell Growth through ERK Activation—To elucidate whether O_2^--mediated ESW promotion of cell proliferation was linked to ERK activation, we found that early scavenging of O_2^- by SOD could suppress ESW-induced ERK activation in 6 h, as demonstrated by specific MBP phosphorylation, and could suppress ESW-promoted osteoprogenitor proliferation in 2 days. Furthermore, we also found that the addition of 20 μM PD98059, an MEK inhibitor, also down-regulated ESW-induced ERK activation (Fig. 6*A*) and resulted in the suppression of ESW promotion of cell growth (Fig. 6*B*). To examine how O_2^- mediated ESW-induced ERK activation, we have further assessed the role of PKC, PKA, and tyrosine kinase in the ESW-induced ERK activation using specific inhibitors. Results showed that the inhibition of PKC with 50 μM calphostain C or

TABLE I

Effect of ESW treatment on the superoxide, nitric oxide, peroxynitrite, TGF-β1 production, and bone nodule formations in bone marrow stromal cells

Rat bone marrow stromal cells in the presence or absence of 500 units/ml SOD, 100 μM urate, or 100 μM L-NAME were treated with or without 0.16 mJ/mm² ESW for 500 impulses. Superoxide (nM O_2^- mg protein/h), nitric oxide (μM NO_2^- + NO_3^-) mg protein/h), nitrotyrosine (nM 3-nitrotyrosine/mg protein/h), TGF-β1 production (pg/ml), and bone nodule formations (bone nodules/well) are as described in the methodologies.

	Control	ESW treatment			
		Vehicle	SOD	Urate	L-NAME
Superoxide	6.2 ± 0.6	16.9 ± 1.4[a]	5.3 ± 1.1[a]	14.4 ± 1.7	18.9 ± 2.6
Nitrotyrosine	9.2 ± 1.9	17.4 ± 1.7[b]	8.6 ± 1.5[b]	4.2 ± 0.8	9.7 ± 1.6
Nitric oxide	10.1 ± 1.4	5.2 ± 0.8[c]	9.1 ± 1.1[c]	5.8 ± 0.9	6.3 ± 1.2
TGF-β1	107.3 ± 18.4	206.8 ± 20.4[d]	121.4 ± 9.8[d]	198.5 ± 18.4	211.4 ± 18.8
Bone nodules	114.6 ± 23.4	316.5 ± 21.2[e]	124.5 ± 19.6[e]	302.8 ± 27.2	297.3 ± 28.5

[a] $p < 0.01$ represents significant differences between both groups indicated. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

[b] $p < 0.004$ represents significant differences between both groups indicated. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

[c] $p < 0.024$ represents significant differences between both groups indicated. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

[d] $p = 0.013$ represents significant differences between both groups indicated. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

[e] $p < 0.001$ represents significant differences between both groups indicated. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

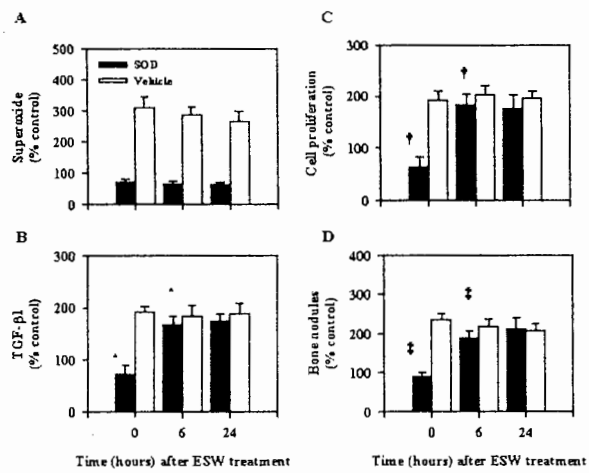

FIG. 5. **Scavenging of O_2^- production by SOD suppressed ESW promotion of osteoprogenitor cell proliferation.** *A*, addition of SOD (500 units/ml) at 0, 6, or 24 h after ESW treatment significantly blocked O_2^- production by bone marrow osteoprogenitor cells. *B*, early addition of SOD at 0 h, but not at 6 or 24 h after ESW treatment, blocked TGF-β1 induction as determined by enzyme-linked immunosorbent assay. *C*, early addition of SOD at 0 h, but not at 6 or 24 h after ESW treatment, blocked osteoprogenitor cell growth as determined by [^{3}H]thymidine incorporation. *D*, early addition of SOD at 0 h, but not at 6 or 24 h after ESW treatment, blocked ESW-induced bone nodule formations as determined by von Kossa staining. Results are presented by mean values ± S.E. calculated from six duplicate experiments as compared with the control without ESW treatment. * ($p < 0.001$), † ($p = 0.023$), and ‡ ($p = 0.017$) represent a significant difference between both groups.

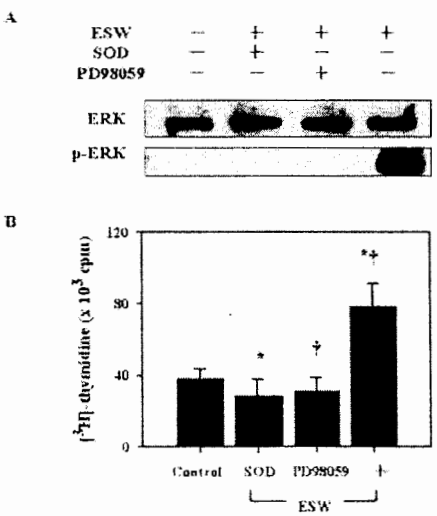

FIG. 6. **Scavenging of O_2^- by SOD and inhibition of ERK by PD98059 suppressed ERK activation and bone marrow stromal cell proliferation.** *A*, addition of SOD or PD98059 blocked ESW-induced ERK activation in 6 h. *Upper lane* shows total ERK protein levels from bone marrow stromal cells with and without SOD or PD98059 treatment on a Western blot. *Lower lane* shows phosphorylated ERK (*p-ERK*) as demonstrated by MBP phosphorylation in 6 h. *B*, addition of SOD or PD98059 blocked ESW promotion of bone marrow stromal cell growth. Cell growth was determined by [^{3}H]thymidine incorporation. * ($p < 0.001$) and † ($p < 0.001$) represent a significant difference between both groups. Results are presented by mean values ± S.E. calculated from six duplicate experiments.

PKA with 100 μM (R_p)-cAMP did not affect ESW-induced ERK activation (Fig. 7*A*) or cell growth (Fig. 7*B*). However, inhibition of tyrosine kinase with 20 μM genistein or blockage of O_2^- production by SOD suppressed ESW-induced ERK activation (Fig. 7*A*) and cell proliferation (Fig. 7*B*). These results suggested that O_2^--mediation of ESW-induced ERK activation comes about through a tyrosine kinase-dependent pathway.

Scavenge of O_2^- by SOD and Inhibition of ERK by PD98059-suppressed ESW-induced Nuclear CBFA1 Phosphorylation and Osteoprogenitor Cell Growth and Maturation—To elucidate the role of O_2^- in the ESW induction of ERK-dependent signal transduction and osteogenesis, we studied whether O_2^- mediated ESW-induced ERK activation and whether specific osteogenic transcription factor, CBFA1 phosphorylation, was linked to an increase of osteocalcin expression and bone nodule formation. It was found that ESW treatment elicited CBFA1 activation, as demonstrated by an increase of phosphorylated CBFA1 in nuclear fraction extracts (Fig. 8*A*). Scavenging of O_2^- by SOD (500 units/ml) significantly suppressed ESW-induced CBFA1 phosphorylation (Fig. 8*A*), and inhibition of ERK activation by PD98059 suppressed ESW-induced CBFA1 activation in 6 h (Fig. 8*A*). Scavenging of O_2^- and inhibiting ERK blocked ESW-promoted osteoprogenitor maturation as demonstrated by osteocalcin production in 12 days (Fig. 8*A*) and bone nodule formations in 21 days (Fig. 8*B*). These results suggest that O_2^- had an important role in the regulation of ERK-mediated osteogenic transcription factor (CBFA1) activation on the ESW promotion of osteoprogenitor cell growth and maturation.

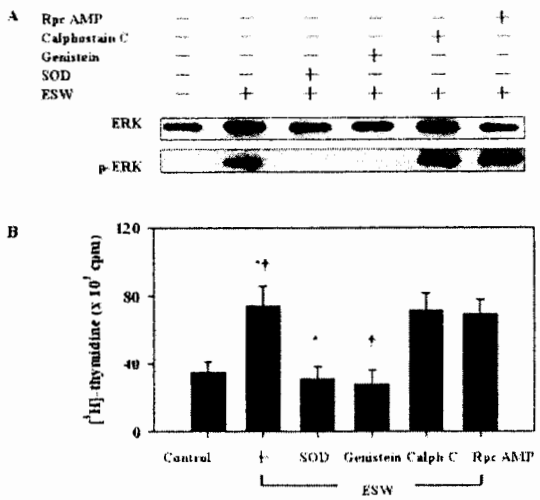

FIG. 7. **Tyrosine kinase, but not PKC or PKA, was involved in ESW promotion of ERK activation and bone marrow stromal cell proliferation.** *A*, addition of SOD or genistein blocked ESW-induced ERK activation. Cells were treated with 500 units/ml SOD, 20 μM genistein, 50 μM calphostain C, or 100 μM (R_p)-cAMP before ESW treatment for 500 impulses. Phosphorylated ERK (*p-ERK*) was determined by Western blot analysis of MBP phosphorylation. *B*, addition of SOD or genistein suppressed ESW-promoted cell growth as demonstrated by [^{3}H]thymidine incorporation. * ($p < 0.001$) and † ($p < 0.001$) represent a significant difference between both groups. Results are presented by mean values ± S.E. calculated from six duplicate experiments.

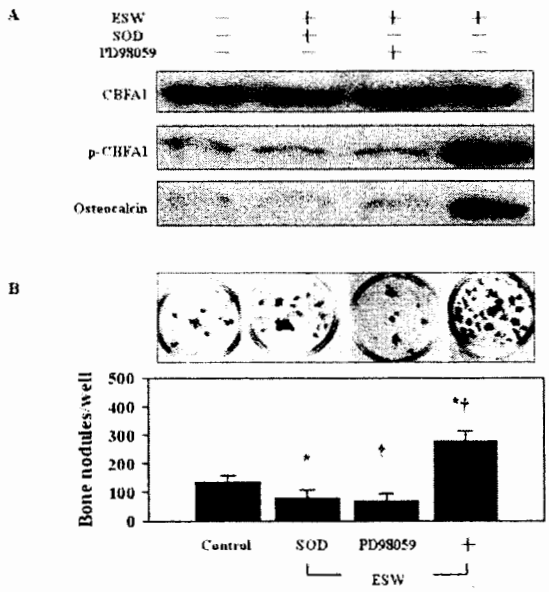

FIG. 8. **Scavenging of O_2^- by SOD and inhibition of ERK by PD98059 suppressed ESW promotion of ERK activation, CBFA1 phosphorylation, osteocalcin, and bone nodule formation.** *A*, addition of SOD or PD98059 blocked ESW-induced CBFA1 activation and osteocalcin expression. *Top lane* shows total CBFA1 protein levels from bone marrow stromal cells with and without ESW treatment in the presence or absence of SOD or PD98059 on a Western blot. *Middle lane* shows the reciprocal CBFA1 activation as demonstrated by tyrosine-phosphorylated CBFA1 (*p-CBFA1*). *Bottom lane* shows the osteocalcin expression. *B*, addition of SOD or PD98059 suppressed ESW-promoted bone nodule formation as determined by von Kossa staining. * ($p <$ 0.001) and † ($p < 0.001$) represent a significant difference between both groups.

DISCUSSION

In an *ex vivo* model, we demonstrated that an optimal ESW treatment of rat femurs could effectively promote CFU-O growth and bone nodule formations from the bone marrow mesenchymal cells. More interestingly, we found that early O_2^- production, followed by higher TGF-β 1 induction was involved in ESW-enhanced CFU-O formation and bone nodule formations since early but not later scavenging of O_2^- by SOD suppressed TGF-β1 production and bone nodule formations. To our knowledge, our finding comprise the first evidence demonstrating that optimal physical ESW treatment can enhance osteoprogenitor cell growth and bone nodule formation via O_2^- induction. Evidence has indicated previously (44) that the biological effect of ESW treatment may be mediated by bubble cavitation or acoustic energy. The ESW-induced acoustic energy could also elicit oxygen free radicals (45). However, the oxidative stress from the ESW treatment has been previously implicated in tissue damage (42). In contrast to previous studies showing that Ca^{2+}, oxidative stress, or PGE_2 was involved in ESW-induced cell injury or mechanical stimulation of osteoblast growth (41, 43, 46), we have shown that O_2^-, but not Ca^{2+}, H_2O_2, or PGE_2, was involved in ESW promotion of bone marrow stromal cell growth.

It has been shown that O_2^- can act as an important signal for the mitogenesis of certain cells (47) and induce cardiac fibroblast proliferation associated with an increase in TGF-β1 expression (48). Inhibition of SOD has been shown to affect cell growth and apoptosis of rat myocytes (49). Recently, Vozenin-Brotons *et al.* (50) demonstrated that SOD acted as a potent antagonist of TGF-β1 expression in superoxide-induced myofibroblast proliferation. TGF-β1 plays an important role in the promotion of osteogenic differentiation of bone marrow stromal cells (23, 51). In addition, TGF-β1 acts an important growth factor in the stimulation of fibrous tissue formation and the regulation of angiogenic growth resulting in tissue regeneration (52). By employing human epithelial alveolar cells, Bellocq *et al.* (53) showed that oxygen and nitrogen free radicals acted through two different mechanisms to regulate TGF-β1 release. Furthermore, oxygen radicals induced by addition of serum are also shown to promote osteoblast cell proliferation (54). Results from our study suggest that physical ESW promotes bone marrow stromal cell growth and differentiation toward osteoprogenitor as a result of early O_2^--mediated TGF-β1 induction.

In contrast to previous studies showing that a large quantity of O_2^- or $ONOO^-$ from skeletal injury or cytotoxic agent was cytotoxic to osteoblasts (55, 56), we have showed that ESW-promoted osteoprogenitor cell growth was mediated by O_2^- production. Superoxide can easily react with NO to generate $ONOO^-$ for the regulation of cell growth at less toxic levels (57). Results in this study demonstrate that ESW-induced osteoprogenitor cell growth is associated with an increase of O_2^- and nitrotyrosine, a marker for $ONOO^-$ formation. Scavenging of $ONOO^-$ by urate did not affect ESW-enhanced TGF-β1 production and bone nodule formation, suggesting that the interaction between O_2^- and NO to generate $ONOO^-$ after ESW treatment is not toxic to osteoprogenitors. In contrast, inhibition of NO production by addition of L-NAME did not suppress ESW-promoted O_2^- production or osteogenesis, indicating that early O_2^-, but not NO, induction is involved in ESW-enhanced osteogenesis.

We have recently suggested (36) that ESW-promoted osteogenesis of bone marrow stromal cells is related to Ras activation. Evidence has also shown that ERK, a member of MAPK family protein kinase, plays an important role in the proliferation and differentiation of mesenchymal stem cells and osteoblastic cells (58–60). Moreover, other studies have also shown that a specific osteogenic transcription factor (CBFA1) activation plays an important role in regulation of osteogenic differentiation in bone marrow stromal cells and calvaria stem cells

(61–63). We have further demonstrated in this study that ESW-induced O_2^- might activate tyrosine kinase but not PKC or PKA to elicit ERK activation. In addition to oxygen radicals, NO induced by shear stress (64), as well as by exogenous NO, was shown to act as a regulatory role in the Ras/ERK pathway activation (65). We, however, found that inhibition of NO production by L-NAME did not alter the ESW-promoted O_2^- production or osteogenesis. Taken together, we have demonstrated that ESW treatment elicits early O_2^- production for tyrosine kinase-mediated ERK activation resulting in CBFA1 phosphorylation for osteoprogenitor cell growth and maturation into bone nodules. Results from these studies suggest that an optimal regulation of redox reactions by biophysical factors such as ESW might provide a promising regimen for the regulation of ERK signal transduction and activation of the specific osteogenic transcription factor, CBFA1, resulting in bone growth. The ESW treatment may not only be applicable for enhancement of osteogenesis in fracture healing and osteoporosis but may also provide an alternative non-invasive method for *ex vivo* extension of mesenchymal stem cells in the future. Moreover, based on the ESW-induced signal transduction pathway for bone growth, the biopharmacological modulation of fracture healing and osteoporosis may also be possible.

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