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/mm² for 500 impulses 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 O₂ but not Ca²⁺, H₂O₂, or prostaglandin E₂ suppressed osteoprogenitor cell growth and maturation. Scavenging of O₂ 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 O₂ acted as an early signal for ESW-induced cell growth. Further studies demonstrated that ESW induced ERK activation, and blockage of O₂ production or inhibition of tyrosine kinase, but not protein kinase A and C inhibitors, suppressed ESW-induced ERK activation. In support that O₂ mediated the ESW-induced ERK activation and osteogenic differentiation, we further demonstrated that scavenging of O₂ 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 O₂ 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 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

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; t-NANE, N-nitro-t-arginine methyl ester; PKA, protein kinase A; PKC, protein kinase C; MBF, myelin basic protein; PGE₂, prostaglandin E₂; 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®). Each rat was placed in supine position with four limbs abducent fixation. The ESW treatment with 0, 250, 50, and 1000 impulses at 0.16 mJ/mm² (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. Cells (nucleated cells) in the bone 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 × 10⁵ cells/well; 24-well plate) was raised in osteogenic medium containing Dulbecco’s modified Eagle medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS), 10⁻⁴ 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% CO₂ 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 histochromogenic 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 × 10⁴ 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 μM glycerine, 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 $A_{405}$mm by a microplate reader (Dyn-Ex Technologies Inc.). Alkaline phosphatase activity was expressed as $μ$mol p-nitrophenol/mg (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 1% silver nitrate for 5 min. The bone nodule formation was determined by high-performance liquid chromatography (LC-10AD; Shimadze, Tokyo, Japan) equipped with a reverse phase column (4.6 mm internal diameter × 250 mm × 5 μm) and a guard cell potential of 400 mV. The nitrotyrosine formation, as described previously (39), 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 O₂⁻ and NO, we measured the nitrotyrosine level, which is a marker for NO-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 × 5 μm). The mobile phase was composed of 50% acetonitrile (ACN) and 50% 0.1 M formic acid (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 (Coulonix 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 standard
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 study. 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-β1-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 A500 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 ([H]thymidine uptake as modified from a measurement of osteoblast proliferation described previously (36). Bone marrow stromal cells with and without ESW treatment (2 × 10^6 cells/well, 96-well plate) were cultured for 24 h before the addition of 1 μCi of ([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 ([H]thymidine uptake determination by a liquid scintillation analyzer (Tri-Carb 2010TR, 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 MgCl2, 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 MgCl2, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% Nonidet P-40, 1% phenylmethylsulfonyl fluoride, 2 μM dithiothreitol, 2 μM leupeptin, and 1 μM 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.

**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 heme strept aunt buffer containing 20 μg of myelin basic protein (MBP), 15 mM MgCl2, 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 phosphophorylated CBFA1 on the blot was further recognized by a specific mouse anti-phospho-tyrosine (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 phosphoplated 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.

**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/mm² 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. 2A). The cells from the CFU-O colonies matured into bone nodules after a 21-day long term culture as shown in Fig. 2B. The bone nodule formations were significantly higher in the ESW treatment with 500 impulses than those without ESW treatment (Fig. 2B).

**ESW Promotion of Osteoprogenitor Cell Growth Mediated by O₂⁻ but Not Ca²⁺, H₂O₂, or PGE₂—** 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 PGE₂ is involved in ultrasound stimulation of mouse osteoblast growth (49). 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 Ca²⁺ with 1 mM EGTA or chelation of intracellular Ca²⁺ with 40 μM BAPTA did not affect the ESW promotion of osteoprogenitor cell growth as demonstrated by [3H]thymidine incorporation (Fig. 3). Scavenging of hydrogen peroxide by 500 μM catalase did not influence ESW-promoted cell growth. Inhibition of COX-2 activity by 10 μM indomethacin, which was a critical enzyme for PGE₂ 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 O₂⁻, but not Ca²⁺,
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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 O₂⁻ production in 1 h (Fig. 4A). This higher production of O₂⁻ persisted for 24 h. To explore whether the O₂⁻ 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. 4B).

ESW-promoted Osteogenesis Mediated by O₂⁻ but Not ONOO⁻—It is well known that O₂⁻ can react rapidly with NO to generate ONOO⁻ (37), which may mediate another signal 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 O₂⁻, NO, or ONOO⁻ was involved in ESW promotion of osteogenesis. As shown in Table I, ESW treatment induced a rapid increase of O₂⁻ and nitrosyls production associated with a decrease in NO production. Addition of SOD significantly decreased ESW-induced O₂⁻ and nitrosyls production but raised the NO level back to the control level (Table I). The addition of urate (100 μM) to scaveng ONOO⁻ did not affect ESW-augmented O₂⁻ or NO levels but significantly suppressed nitrosyls 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 O₂⁻ production, TGF-β1 production, or bone nodule formation (Table I).

Early but Not Late Scavenge of O₂⁻ 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 O₂⁻ acted an early or late signal for bone marrow mesenchymal cell growth. As shown in Fig. 4A, the addition of SOD at 0, 6, or 24 h after ESW treatment effectively scavenged ESW-induced O₂⁻ 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 O₂⁻ production, did not significantly suppress TGF-β1 production (Fig. 5B). Similarly, the early addition of SOD at 0 h, but not at 6 or 24 h, suppressed ESW-promoted cell growth as demonstrated by [³H]thymidine incorporation (p < 0.001) (Fig. 5C). Moreover, it was found that the early addition of SOD did significantly suppress the ESW-augmented bone nodule formations (Fig. 5D).

ESW-promoted Cell Growth through ERK Activation—To elucidate whether O₂⁻-mediated ESW promotion of cell proliferation was linked to ERK activation, we found that early scavenging of O₂⁻ by SOD could suppress ESW-induced ERK activation in 6 h, as demonstrated by specific MAPK phospho-tyrosine, 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. 6A) and resulted in the suppression of ESW promotion of cell growth (Fig. 6B). To examine how O₂⁻ mediated ESW-induced ERK activation, we have further assessed the role of PKC, P38, 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...
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TABLE I

| Treatment | Control | ESW | l-NAME |
|-----------|---------|-----|--------|
| Superoxide | 6.2 ± 0.6 | 16.9 ± 1.4<sup>a</sup> | 5.3 ± 1.1<sup>b</sup> | 14.4 ± 1.7 |
| Nitrotyrosine | 9.2 ± 1.9 | 17.4 ± 1.7<sup>e</sup> | 8.6 ± 1.5<sup>c</sup> | 4.2 ± 0.8 |
| Nitric oxide | 10.1 ± 1.4 | 5.2 ± 0.8<sup>b</sup> | 9.1 ± 1.1 | 5.8 ± 0.9 |
| TGF-β1 | 107.3 ± 18.4 | 206.8 ± 20.4<sup>e</sup> | 121.4 ± 9.8<sup>c</sup> | 198.5 ± 18.4 |
| Bone nodules | 114.6 ± 23.4 | 316.5 ± 21.2<sup>e</sup> | 124.5 ± 19.6<sup>c</sup> | 302.8 ± 27.2 |

<sup>a</sup>p < 0.01 represents significant differences between both groups. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

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

<sup>c</sup>p = 0.024 represents significant differences between both groups indicated. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

<sup>d</sup>p = 0.013 represents significant differences between both groups indicated. Results are presented with mean values ± S.E. calculated from six paired triplicate experiments.

<sup>e</sup>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 O₂⁻ 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 O₂⁻ 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 [³H]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.

PKA with 100 µM (R,</sup><sup>e</sup>p)-cAMP did not affect ESW-induced ERK activation (Fig. 7A) or cell growth (Fig. 7B). However, inhibition of tyrosine kinase with 20 µM genistein or blockage of O₂⁻ production by SOD suppressed ESW-induced ERK activation (Fig. 7A) and cell proliferation (Fig. 7B). These results suggested that O₂⁻-mediation of ESW-induced ERK activation comes about through a tyrosine kinase-dependent pathway.

Scavenging of O₂⁻ by SOD and Inhibition of ERK by PD98059—To elucidate the role of O₂⁻ in the ESW induction of ERK-dependent signal transduction and osteogenesis, we studied whether O₂⁻ 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. 8A). Scavenging of O₂⁻ by SOD (500 units/ml) significantly suppressed ESW-induced CBFA1 phosphorylation (Fig. 8A), and inhibition of ERK activation by PD98059 suppressed ESW-induced CBFA1 activation in 6 h (Fig. 8A). Scavenging of O₂⁻ and inhibiting ERK blocked ESW-promoted osteoprogenitor maturation as demonstrated by osteocalcin production in 12 days (Fig. 8A) and bone nodule formations in 21 days (Fig. 8B). These results suggest that O₂⁻ 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. 6. Scavenging of O₂⁻ 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 cell growth. Cell growth was determined by [³H]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.

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 ml/mm² ESW for 500 impulses. Superoxide (500 units mg protein/h), nitric oxide (100 µM NO₂⁻ + NO₃⁻) mg protein/h), nitrotyrosine (8.6 mg protein/h), and Osteoprogenitor Cell Growth and Maturation—To elucidate the role of O₂⁻ in the ESW induction of ERK-dependent signal transduction and osteogenesis, we studied whether O₂⁻ 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. 8A). Scavenging of O₂⁻ by SOD (500 units/ml) significantly suppressed ESW-induced CBFA1 phosphorylation (Fig. 8A), and inhibition of ERK activation by PD98059 suppressed ESW-induced CBFA1 activation in 6 h (Fig. 8A). Scavenging of O₂⁻ and inhibiting ERK blocked ESW-promoted osteoprogenitor maturation as demonstrated by osteocalcin production in 12 days (Fig. 8A) and bone nodule formations in 21 days (Fig. 8B). These results suggest that O₂⁻ 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.
growth and bone nodule formations from the bone marrow mesenchymal cells. More interestingly, we found that early $O_2^-$ production, followed by higher TGF-$\beta$-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-$\beta$-1 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$_2$O$_2$, 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-$\beta$-1 expression (48). Inhibition of SOD has been shown to affect cell growth and apoptosis of rat myocytes (49). Recently, Vozenin-Brottons et al. (50) demonstrated that SOD acted as a potent antagonist of TGF-$\beta$-1 expression in superoxide-induced myofibroblast proliferation. TGF-$\beta$-1 plays an important role in the promotion of osteogenic differentiation of bone marrow stromal cells (23, 51). In addition, TGF-$\beta$-1 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-$\beta$-1 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-$\beta$-1 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-$\beta$-1 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.

**DISCUSSION**

In an ex vivo model, we demonstrated that an optimal ESW treatment of rat femurs could effectively promote CFU-O
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(61–63). We have further demonstrated in this study that ESW-induced O$_2^\cdot$ might activate tyrosine kinase but not PKC or PTKA 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). However, we found that inhibition of NO production by l-NAME did not alter the ESW-promoted O$_2^\cdot$ production or osteogenesis. Taken together, we have demonstrated that ESW treatment elicits early O$_2^\cdot$ 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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