8-Nitro-cGMP suppresses mineralization by mouse osteoblasts

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Nitric oxide and reactive oxygen species regulate bone remodeling, which occurs via bone formation and resorption by osteoblasts and osteoclasts, respectively. Recently, we found that 8-nitro-cGMP, a second messenger of nitric oxide and reactive oxygen species, promotes osteostogenesis. Here, we investigated the formation and function of 8-nitro-cGMP in osteoblasts. Mouse calvarial osteoblasts were found to produce 8-nitro-cGMP, which was augmented by tumor necrosis factor-α (10 ng/ml) and interleukin-1β (1 ng/ml). These cytokines suppressed osteoblastic differentiation in a NO synthase activity-dependent manner. Exogenous 8-nitro-cGMP (30 μmol/L) suppressed expression of osteoblastic phenotypes, including mineralization, in clear contrast to the enhancement of mineralization by osteoblasts induced by 8-bromo-cGMP, a cell membrane-permeable analog of cGMP. It is known that reactive sulfur species denitrates and degrades 8-nitro-cGMP. Mitochondrial cysteinyl-IRNA synthetase plays a crucial role in the endogenous production of RSS. The expression of osteoblastic phenotypes was suppressed by not only exogenous 8-nitro-cGMP but also by silencing of the Cars2 gene, indicating a role of endogenous 8-nitro-cGMP in suppressing the expression of osteoblast phenotypes. These results suggest that 8-nitro-cGMP is a negative regulator of osteoblastic differentiation.

Key Words: nitric oxide, 8-nitro-cGMP, osteoblasts, differentiation, mineralization

Bone tissue is metabolically active and continuously remodeled by resorption of old bone tissue by osteoclasts differentiated from monocyte/macrophage lineage cells, followed by the formation of new bone tissue by osteoblasts derived from mesenchymal stem cells. Mechanical strength and the mass and shape of individual bones are maintained by a dynamic equilibrium between bone resorption and bone formation, and an imbalance between these processes causes pathological changes in bone tissues, including osteoporosis and osteopenosis.(1)

Previous investigations have suggested that nitric oxide (NO) and reactive oxygen species (ROS) positively regulate osteoclast differentiation and bone resorption.(2–6) Recently, we found that 8-nitro-cGMP, a second messenger of NO and ROS,(10–12) is generated in osteoclasts and macrophages in a manner dependent on NO synthase (NOS) and NADPH oxidase and promotes receptor activator of nuclear factor-κ-B (RANKL) ligand (RANKL)-induced differentiation of osteoclasts.(13) This study also showed that interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) augment the production of 8-nitro-cGMP in osteoclasts, indicating that 8-nitro-cGMP may contribute, at least in part, to the inflammation-related reduction of bone mass with enhanced bone resorption by osteoclasts.

It has been reported that NO and cGMP promote bone formation,(14) whereas inflammatory cytokines such as IL-1β and TNF-α suppress osteoblast differentiation.(15–17) One of those studies showed that TNF-α in combination with IL-1β induces expression of the inducible NO synthase (NOS) (NOS2) gene and suppresses osteoblastic differentiation in a NOS activity-dependent manner.(15) Hence, it can be said that NO has a double-sided activity toward the bone formation. It is also known that osteoblastic differentiation is down-regulated by oxidative stress.(18–21) Together, these observations suggest that, in contrast to the enhancing effects of NO and ROS on osteoclastogenesis, osteoblastic differentiation is suppressed by NO in the presence of ROS in inflammatory conditions. In the present study, we investigated the role of 8-nitro-cGMP in the expression of osteoblastic phenotypes in mouse osteoblasts and found that it suppresses mineralization by osteoblasts.

Materials and Methods

Cell cultures. We purchased MC3T3-E1 cells, a cell line derived from newborn mouse calvarial osteoblasts, from the Riken BioResource Center (Tsukuba, Japan). Mouse primary osteoblasts were isolated from calvaria of newborn ddY mice provided by Japan SLC Inc. (Hamamatsu, Japan) using a conventional method,(22) according to a protocol approved by the ethical board for animal experiments of Showa University (approval number 17055). Both MC3T3-E1 cells and mouse primary osteoblasts were cultured in αMEM (Fujifilm Wako Pure Chemical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), penicillin G, streptomycin, and amphotericin B at 37°C in a humidified atmosphere of 5% CO2/95% air. The proliferation of the cells was assessed by counting the viable cells after trypsinization and staining with trypan blue.

Reagents. Recombinant human bone morphogenetic protein-2 (BMP-2), recombinant mouse IL-1β, and TNF-α were purchased from R&D Systems (Minneapolis, MN). 8-Bromo-cGMP was obtained from Sigma Aldrich (St. Louis, MO). 8-Nitro-cGMP was prepared according to a previously reported method.(10) All other reagents used in this study were from commercial sources.

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**Introduction of small interfering (si)RNA.** Stealth™ siRNAs (Invitrogen, Carlsbad, CA) for mouse Nos2 and Cars2 (mitochondrial cysteinyl-rRNA synthetase gene) and a negative control siRNA were obtained from Invitrogen (Carlsbad, CA). Each siRNA (30 pmol) was introduced into cells at 40–50% confluence grown in antibiotic/antimycotic-free αMEM containing 10% FBS using Lipofectamine™ RNAiMAX (Invitrogen) by reverse transfection. After 24 h incubation, the culture medium was changed to fresh containing antibiotics/antimycotic. Reduced expression of each target gene was confirmed using a real-time reverse transcription (RT)-polymerase chain reaction (PCR) method.

**RT-PCR and real-time RT-PCR.** Total RNA was extracted from cells cultured in 6-well plates using TRIzol™ Reagent (Life Technologies, Carlsbad, CA). RT reactions were performed using ReverTra Ace RT qPCR master mix (TOYOBO Co. Ltd., Osaka, Japan) and a random hexamer. PCR assays for Nos2 and Gapdh were performed using the following primers: Nos2, 5'-ACG TTG GGT TCT TGT TCA CT-3' and 5'-GTC TCT GGC TCT GGT CA-3' (468 bp); Gapdh, 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CAT TA-3' (452 bp). Quantitative real-time PCR assays were performed using a TaqMan™ Gene Expression Assay kit (Life Technologies) and StepOne Real-time PCR System (Applied Biosystems, Foster City, CA). Amplification signals from the target genes were normalized against mouse glyceraldehyde 3-phosphatase (Gapdh) (assay ID: Mm99999915_g1) and expressed as relative values. The assay IDs for mouse tissue-nonspecific alkaline phosphatase (Tnap), osteocalcin/bone Gla protein (Bglap), and Cars2 were Mm00475834_m1, Mm03431826_mH, and Mm01209062_m1, respectively.

**Assessment of alkaline phosphatase (ALP) activity.** Cells were plated in 96-well plates at a density of 1 × 10⁴ cells/well, then cultured for 72 h in the above-mentioned medium containing BMP-2 (100 ng/ml), in the presence and absence of either 8-nitro-cGMP or 8-bromo-cGMP (30 μmol/L). Cells were fixed for 30 min in 4% parafomaldehyde and washed with PBS, then incubated for 30 min at 37°C with 100 μmol/L Tris-HCl buffer (pH 8.5) containing 270 μmol/L naphthol AS-MX phosphate (Sigma-Aldrich) and 1.4 mmol/L Fast blue BB (Sigma-Aldrich). After washing with tap water, they were observed under a microscope.

Cells were cultured in the same conditions described above, washed with PBS, and homogenized with 1% Nonidet P-40 (50 μl) under sonication on ice. Cell lysates (10 μl) were added to 50 μl of 0.2 mol/L Tris–HCl buffer (pH 9.5) containing one mmol/L MgCl₂ and 12.5 mmol/L disodium p-nitrophenyl phosphate (Fujifilm Wako Pure Chemical Co.). After incubation for 15 min at 37°C, the reactions were terminated by adding 50 μl of 0.5 mol/L NaOH, and the absorbance of the reaction mixture at 405 nm was read using a microplate reader (SH-1000; Corona Electric, Ibaraki, Japan). The increase in absorbance after 15 min was divided by the amount of cellular protein, with the obtained value used to express the specific activity of ALP.

**Visualization of mineralized nodules by Alizarin red staining.** Cells were plated in 384-well plates at a density of 2.5 × 10⁴ cells/well or 96-well plates at a density of 1 × 10⁴ cells/well, and cultured for 5 or 7 days in the medium as described above, supplemented with BMP-2 (100 ng/ml), ascorbic acid (50 μg/ml), β-glycerophosphate (10 mmol/L), and dexamethasone (10 mmol/L), in the presence or absence of various concentrations of 8-nitro-cGMP. Next, the cells were washed with PBS, fixed in 95% methanol, stained with 1% Alizarin red S (pH 6.3–6.5) (Fujifilm Wako Pure Chemical Co.) for 5 min, and washed several times with water. After observation under a microscope, Alizarin red S was dissolved in 10% (w/v) cetylpyridinium chloride in water and quantified by reading absorbance at 570 nm.

**Western blot analysis.** Cells were cultured for 12 h in 6-well plates at a density of 1 × 10⁴ cells/well in the presence or absence of 8-nitro-cGMP (30 and 100 μmol/L). Cells were washed twice with PBS and collected using a scraper, then lysed in RIPA buffer (Fujiﬁlm Wako Pure Chemical Co.). Cells lysates (20 μg of protein) were subjected to SDS-PAGE (10% acrylamide) under reducing conditions, then separated proteins were transferred onto PVDF membranes and immunoblotted using an anti-RS-CGP antibody (90) and β-actin. Immunoreactive bands were visualized by an enhanced chemiluminescence reaction with an ECL Prime Western Blot Detection System (GE Healthcare, Chicago, IL). The intensity of whole bands detected by anti-RS-cGMP antibody and that by anti-β-actin antibody was quantified using Versa Doc 5000 MP (BioRad Laboratories, Hercules, CA).

**Immunocytochemical detection of 8-nitro-cGMP.** Immunocytochemical detection of 8-nitro-cGMP was performed as previously described.(12,23) Briefly, cells in 96-well plates were ﬁxed in Zamboni's solution (0.1 mol/L sodium phosphate buffer, pH 7.4) containing 4% (w/v) paraformaldehyde and 2 mg/ml 2,4,6-trinitrophenol (picric acid), then incubated overnight with the IgG anti-8-nitro-cGMP monoclonal antibody (Primary antibodies bound to 8-nitro-cGMP were observed under a fluorescence microscope (Keyence, Osaka, Japan) after visualization by incubation with Alexa Fluor™568-labeled goat anti-mouse IgG (Abcam, Cambridge, UK).

**Quantification of 8-nitro-cGMP incorporated by osteoblasts.** For quantification of 8-nitro-cGMP incorporated in osteoblasts cultured in the presence of exogenous 8-nitro-cGMP, mouse calvarial osteoblasts (8.0 × 10⁴ cells/well) were plated in 6-well plates and cultured overnight in αMEM containing 10% FBS. The medium was changed with the fresh containing 0 or 30 μmol/L 8-nitro-cGMP. After incubation for 48 h at 37°C in a CO₂ incubator, cells were washed three times with PBS (2 ml) and scraped in 88% methanol containing 5 mmol/L N-ethylmaleimide (NEM), 20 mmol/L [¹⁴C]8-nitro-cGMP, and 2% acetic acid. After centrifugation for 5 min at 14,000 rpm, the supernatants were applied to 1-cc wax columns equilibrated with methanol. After washing with methanol containing 2% acetic acid (0.5 ml) and methanol (0.5 ml), nucleotides were eluted by methanol containing 15% ammonia (0.5 ml). The eluates were dried in vacuo and dissolved in 0.1% formic acid (200 μl) containing 50 mmol/L [¹⁴C]8-nitro-cGMP. The extracts were applied to liquid chromatography (LC) coupled tandem mass spectrometry (MS/MS) to quantify 8-nitro-cGMP. Cell precipitates were dissolved in PBS containing 1% SDS and used to determine protein content by the bicinchoninic acid assay method.

**Statistical analysis.** Values are expressed as the mean ± SD. Mann-Whitney’s U test, Dunnett’s test, and a Steel-Dwass test were used for statistical analyses, with p values less than 0.05 considered to indicate statistical significance.

**Results**

**Enhanced production of 8-nitro-cGMP and suppressed expression of osteoblast marker genes in osteoblasts co-stimulated with IL-1β and TNF-α.** Mouse primary osteoblasts cultured in the presence of BMP-2 (100 ng/ml) produced 8-nitro-cGMP, which was augmented by co-stimulation with IL-1β (1 ng/ml) and TNF-α (10 ng/ml). Production of 8-nitro-cGMP production in osteoblasts treated with IL-1β and TNF-α was suppressed by 5 mmol/L N⁶-nitro-L-arginine methyl ester hydrochloride (L-NAME), a pan-NOS inhibitor (Fig. 1). Since NOS2 expression and NO production in osteoblasts are upregulated by stimulation with TNF-α, especially in combination with IL-1β(9,10) or IL-17,(24,25) the enhanced NO production is likely to cause an increase in the production of 8-nitro-cGMP in osteoblasts. Also, it is known that TNF-α suppresses osteoblastic
Fig. 1. Effects of IL-1β and TNF-α on the production of 8-nitro-cGMP and expressions of osteoblast marker genes in mouse osteoblasts. (A) Mouse calvarial osteoblasts were exposed to IL-1β (1 ng/ml) and TNF-α (10 ng/ml) for 24 h in the absence (−) and presence (+) of L-NAME (5 mmol/L), after which 8-nitro-cGMP was detected immunocytochemically using a specific antibody (red). Bars, 100 μm. (B) The effects of the introduction of Nos2 siRNA and treatments with IL-1β (1 ng/ml) and TNF-α (10 ng/ml) on the expression of Nos2 mRNA in osteoblasts were examined by RT-PCR, with Gapdh expression used as a control. (C, D) The effects of the introduction of Nos2 siRNA and treatments with IL-1β (1 ng/ml) and TNF-α (10 ng/ml) on expressions of Tnap mRNA (C) and Bglap mRNA (D) in osteoblasts were evaluated by real-time RT-PCR, with the expression levels of those genes normalized to that of Gapdh. Results are expressed as relative to the control culture (far left columns). Values are shown as the mean ± SD of 4 cultures. *Significant difference (p<0.05). See color figure in the on-line version.

On the other hand, the same concentration of 8-nitro-cGMP suppressed ALP activity (Fig. 3A), mineralization of the extracellular matrix (Fig. 3B), and expression of Bglap mRNA (Fig. 3C) in the osteoblast cultures. It was a clear contrast that 8-bromo-cGMP, a membrane-permeable analog of cGMP, enhanced ALP activity and mineralization in cultures of mouse primary osteoblasts (Fig. 3D and E), which were consistent with previous reports that noted a positive role for cGMP in osteoblast differentiation.[12,26,27]

Suppressed differentiation and mineralization in osteoblasts by silencing Cars2 gene. It is known that 8-nitro-cGMP undergoes sulphydrylation by hydrogen sulfide and far more efficiently by cysteine hydrosulfide (CysSSH) and CysSSH-derived persulfides and polysulfides, molecules generally termed as reactive sulfur species (RSS), and results in the formation of 8-SH-cGMP.[10,12,25,29] While 8-SH-cGMP still has an activity to activate cGMP-dependent kinase, it is not able to S-guanylate the sulphydryl group of proteins. The sulphydryl group of 8-SH-cGMP is removed in the presence of NO and ROS. The resultant cGMP is hydrolyzed into GMP by phosphodiesterases.[10,12,25,29] Hence, endogenous production of RSS, including CysSSH and CysSSH-derived persulfides and polysulfides is considered critical for the degradation of intracellular 8-nitro-cGMP. It was reported that mitochondrial cysteiny1-tRNA synthetase/CysSSH synthase (CARS2/CERS) has an activity to synthesize CysSSH with L-cysteine used as the substrate.[29] We, therefore, examined the effects of silencing the Cars2 gene (Fig. 4A) on the expression of osteoblastic phenotypes in mouse osteoblasts cultured in

differentiation[15-17] in a NOS2 activity-dependent manner.[15] Hence, we examined the effects of the introduction of siRNA to the Nos2 gene (Fig. 1B) on BMP-2-induced expression of mRNAs for Tnap and Bglap, osteoblast marker genes, in mouse primary osteoblasts following exposure to IL-1β and TNF-α. Expression of these genes was nearly wholly abolished in the presence of IL-1β and TNF-α and significantly recovered by Nos2 siRNA (Fig. 1C and D), indicating that NO produced by NOS2 plays a pivotal role in the suppressed expression of osteoblastic phenotypes under an inflammatory condition.

Suppressed expression of osteoblast markers by 8-nitro-cGMP. Our findings of enhanced production of 8-nitro-cGMP and the suppressed expression of Tnap and Bglap in osteoblasts exposed to IL-1β and TNF-α suggested the possibility that 8-nitro-cGMP participates in reduced expression of osteoblastic phenotypes. Hence, we examined the effects of exogenous 8-nitro-cGMP on ALP activity, mineralization, and Bglap expression in primary osteoblasts cultured in the presence of BMP-2. Incorporation of the 8-nitro-cGMP by mouse calvarial osteoblasts was confirmed by quantifying the amounts of 8-nitro-cGMP in osteoblasts by LC-MS/MS after treatment with exogenous 8-nitro-cGMP (Fig. 2A and B). The intracellular 8-nitro-cGMP significantly increased after 48-h exposure to 30 μmol/L 8-nitro-cGMP (Fig. 2B). The incorporation of 8-nitro-cGMP added to the culture medium was also confirmed by the evidence of the enhanced formation of S-guanylated proteins in osteoblasts (Fig. 2C and D). Exogenous 8-nitro-cGMP (30 μmol/L) did not affect osteoblast proliferation (Fig. 2E).
the presence of BMP-2 to elucidate the role of endogenously produced 8-nitro-cGMP. Western blot analysis revealed that the amounts of S-guanylated proteins in the cells were increased after the introduction of Cars2 siRNA (Fig. 4B), indicating that silencing of the Cars2 gene caused an increase in endogenous 8-nitro-cGMP in osteoblasts. Under that condition, ALP activity and mineralization in osteoblast cultures were significantly suppressed (Fig. 4C and D). In addition, the expression of Bglap was also down-regulated by Cars2 siRNA (Fig. 4E). These results suggest that not only exogenous but also endogenous 8-nitro-cGMP suppresses the expressions of osteoblastic phenotypes. Contrary to 8-nitro-cGMP, 8-bromo-cGMP enhanced the expression of ALP and mineralization in osteoblast cultures (Fig. 3D and E), which was consistent with the previous studies. It was reported that the enhanced expression of osteix, one of the transcription factors required for osteoblast differentiation and maturation, is a possible mechanism of the positive effects of the cGMP-protein kinase G (PKG) system on osteoblast differentiation. The present results indicate that cGMP-PKG signals cannot explain the suppressed osteoblastic phenotypes in osteoblasts treated with 8-nitro-cGMP. While further studies are required to elucidate how 8-nitro-cGMP acts in osteoblasts, the functions of 8-nitro-cGMP other than the activation of PKG, including S-guanylation of proteins, are possibly involved in the suppressed osteoblast differentiation and deposition of calcium phosphate in the extracellular matrix.

The intracellular 8-nitro-cGMP in osteoblasts treated with the exogenous 8-nitro-cGMP (30 μmol/L) reached around 15 to 20 pmol/mg cellular protein in 48 h (Fig. 2A and B). If we assume that the volume and protein content per cell are 1 μL and 1 ng protein/cell, then the intracellular concentration of 8-nitro-cGMP is considered 15–20 μmol/L. A similar range of endogenous 8-nitro-cGMP was observed in other experimental systems, including rat glioma cells stimulated by lipopolysaccharide and...
Inflammatory cytokines\cite{11} and mouse brain\cite{12}. Hence, our experimental conditions using the exogenous 8-nitro-cGMP are considered biologically possible. It is plausible that 8-nitro-cGMP, with extracellular and intracellular concentrations in the range of several micromolar, caused inhibition of osteoblast differentiation and mineralization.

Several studies of the roles of NO in osteoblast differentiation and bone formation with treatment for osteoporosis with NO donors have been presented. Clinical pilot studies have shown that NO donors ameliorate bone formation in osteoporotic patients\cite{33,34}. Furthermore, the involvement of NO and cGMP in osteoblast differentiation has also been reported\cite{14,26,27,33}. On the other hand, some have suggested that the effect of NO on bone formation is biphasic, with a higher level of NO suppressing bone formation\cite{35,36}. In addition to NO, oxidative stress is an important determinant of bone density, with a negative correlation found between bone density and total oxidative/anti-oxidative status in osteoporotic patients\cite{37}. It was also reported that TNF-α in combination with IL-1β induced NOS2 expression and NO production, which play an important role in suppressing osteoblastic differentiation by those inflammatory cytokines\cite{13}.

Our finding that 8-nitro-cGMP, a second messenger of NO and ROS, suppresses the expressions of osteoblastic phenotypes may explain the negative regulation of osteoblastic differentiation by excessive levels of NO and ROS.

CARS2/CPERS was recently shown to be a major enzyme responsible for the production of CysSSH\cite{30}. Suppressed expression of osteoblastic phenotypes, including mineralization in cultured osteoblasts by incorporation of Cars2 siRNA (Fig. 4), is consistent with our finding that exogenous 8-nitro-cGMP induced similar changes in osteoblasts (Fig. 3). Thus, reduced production of CysSSH might result in reduced amounts of RSS in osteoblasts. Furthermore, since RSS is regarded as the significant executioner of degradation of 8-nitro-cGMP\cite{28,29}, it is plausible that silencing of the Cars2 gene suppresses the degradation of intracellular 8-nitro-cGMP produced endogenously in osteoblasts and enhances suppressed expression of osteoblastic phenotypes by endogenous 8-nitro-cGMP.

In a previous study, we noted that 8-nitro-cGMP promotes osteoclast differentiation\cite{13} while the present results suggest that 8-nitro-cGMP is a negative regulator of osteoblastic differentiation. Considering that formation of 8-nitro-cGMP in both osteo-b
clasts and osteoblasts was shown to be augmented by inflammatory cytokines, it seems reasonable to conclude that 8-nitro-cGMP participates in inflammation-related bone loss by enhancing bone resorption by osteoclasts and suppressed bone formation by osteoblasts.

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Abbreviations

BMP-2  bone morphogenetic protein-2  
CARS2  mitochondrial cysteiny-l-tRNA synthetase  
CPERS  cysteine persulfide synthase  
CysSSH  cysteine hydrosulfide  
NO  nitric oxide  
NOS  NO synthase  
PCR  polymerase chain reaction  
ROS  reactive oxygen species  
RSS  reactive sulfur species  
RT  reverse transcription  
siRNA  small interfering RNA

Conflict of Interest

No potential conflicts of interest were disclosed.
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