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Reactive oxygen species derived from Nox4 mediate *BMP2* gene transcription and osteoblast differentiation

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Abstract

BMP-2 (bone morphogenetic protein-2) promotes differentiation of osteoblast precursor cells to mature osteoblasts that form healthy bone. In the present study, we demonstrate a novel mechanism of BMP-2-induced osteoblast differentiation. The antioxidant NAC (*N*-acetyl-L-cysteine) and the flavoprotein enzyme NAD(P)H oxidase inhibitor DPI (diphenyleneiodonium) prevented BMP-2-stimulated alkaline phosphatase expression and mineralized bone nodule formation in mouse 2T3 pre-osteoblasts. BMP-2 elicited a rapid generation of ROS (reactive oxygen species) concomitant with increased activation of NAD(P)H oxidase. NAC and DPI inhibited BMP-2-induced ROS production and NAD(P)H oxidase activity respectively. NAD(P)H oxidases display structurally similar catalytic subunits (Nox1–5) with differential expression in various cells. We demonstrate that 2T3 pre-osteoblasts predominantly express the Nox4 isotype of NAD(P)H oxidase. To extend this finding, we tested the functional effects of Nox4. Adenovirus-mediated expression of dominant-negative Nox4 inhibited BMP-2-induced alkaline phosphatase expression. BMP-2 promotes expression of *BMP2* for maintenance of the osteoblast phenotype. NAC and DPI significantly blocked BMP-2-stimulated expression of *BMP2* mRNA and protein due to a decrease in *BMP2* gene transcription. Dominant-negative Nox4 also mimicked this effect of NAC and DPI. Our results provide the first evidence for a new signalling pathway linking

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AUTHOR CONTRIBUTION

Chandi Mandal performed all of the experiments in the paper. Suthakar Ganapathy assisted in performing confocal microscopy to determine ROS production. Yves Gorin and Hanna Abboud provided intellectual input. Karen Block and Kalyankar Mahadev developed reagents to study Nox4. Stephen Harris developed the BMP-2 flx/flx mice. Goutam Ghosh-Choudhury helped in analysing and arranging the data, and jointly wrote the manuscript with Nandini Ghosh-Choudhury. Nandini Ghosh-Choudhury developed the project and formulated experimental designs to confirm this novel hypothesis.

BMP-2-stimulated Nox4-derived physiological ROS to BMP-2 expression and osteoblast differentiation.

Keywords

bone morphogenetic protein-2 (BMP-2); bone morphogenetic protein-2 gene autoregulation; bone morphogenetic protein-2 signalling; osteoblast differentiation; reactive oxygen species

INTRODUCTION

Bone remodelling is regulated by the neuro-endocrine signalling interplay among several cytokines, hormones, and growth and differentiation factors [1,2]. Balanced activities of osteoblasts and osteoclasts, and the cross-talk between these two cell types, contribute to healthy bone formation [3]. The role of BMP-2 (bone morphogenetic protein-2) in osteoblast differentiation is well established. We and others have shown that BMP-2 regulates timely expression of the genes required for osteoblasts and osteoblast-assisted osteoclast differentiation and activity [4–8]. There are two critical regulatory nodes in this highly co-ordinated process: (i) the cross-talk of different signalling pathways regulated by BMP-2 and (ii) the activation of transcription factors by these signalling molecules to induce appropriate genes and subsequent protein expression.

BMP-2 recruits BMPR I (type I BMP receptor) and BMPR II (type II BMP receptor) to initiate signal transduction [9–11]. Activated BMPR I phosphorylates receptor-specific Smads 1, 5 and 8, which form complexes with the common Smad, Smad4. The heterodimer translocates to the nucleus and stimulates transcription of the BMP-induced genes containing active Smad-binding elements [12,13]. We have previously reported that BMP-2-stimulated non-Smad signalling contributes to osteoblast differentiation. We have shown that BMP-2 activates PI3K (phosphoinositide 3-kinase) and its downstream target Akt. This lipid kinase cascade co-operates with Smad1/5 to induce osteoblast differentiation and osteoblast-assisted osteoclastic gene expression [14,15]. In the present study we provide the first evidence for activation of another novel non-Smad signalling pathway by BMP-2.

ROS (reactive oxygen species) have been implicated in initiation and progression of many human diseases [16,17]. Generation of superoxide anion in diseased states is predominantly a natural by-product of normal oxidative phosphorylation in mitochondria, and is produced by complex I and complex III of the electron transport chain [18,19]. Superoxide is readily dismutated to hydrogen peroxide by superoxide dismutase [20]. Although excessive ROS generation is associated with pathology of many diseases, including bone loss [21], low levels of ROS exhibit a physiological intracellular signalling role, leading to proliferation [22], migration [23], apoptosis [24] and differentiation [25,26]. The physiological ROS are generated predominantly by NAD(P)H oxidases at the plasma membrane and endomembranes [19]. Five isoforms of NAD(P)H oxidase (Nox1–5) have been identified including the classical neutrophil gp91^{phox} (Nox2) [16,27,28]. The role of ROS in osteoclast differentiation has been previously described [29–31]. RANKL [receptor activator of NF- κ B (nuclear factor κ B) ligand] induces Nox-derived ROS as an essential mechanism for osteoclast differentiation [29–31]. However, the involvement of NAD(P)H oxidase in

osteoblast differentiation has not been investigated. In the present study, we examined the contribution of ROS in BMP-2-induced osteoblast differentiation. We identify Nox4 as the predominant NAD(P)H oxidase expressed in the osteoblast that contributes to its differentiation in response to BMP-2.

MATERIALS AND METHODS

Materials

Recombinant BMP-2 and anti-BMP-2 antibody were provided by Wyeth. Tissue culture reagents and one-step RT (reverse transcription)–PCR kit were obtained from Invitrogen. The luciferase assay kit was purchased from Promega. Anti-actin antibody, TRI Reagent for RNA isolation, DPI (diphenyleneiodonium), NAC (*N*-acetyl-L-cysteine) and Alizarin Red S were purchased from Sigma. DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) was obtained from Molecular Probes. SYBR green PCR mix was purchased from Superarray Biosciences.

Cells and Ad (adenovirus) infection

The murine 2T3 pre-osteoblast cells and 2T3 cells [32] stably transfected with the 2.7 kb mouse BMP-2 promoter-driven firefly luciferase expression plasmid (2T3–Luc cells) [32,33] were cultured in α MEM (α -minimal essential medium), supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ in air. Ad vectors expressing wild-type (WT Nox4) and mutant human Nox4 with deletion of NAD(P)H- and FAD-binding domains (Ad- N/ F Nox4) have been described previously [34] and were kindly provided by Dr B.J. Goldstein (Merck Research Laboratories, Rahway, NJ, U.S.A.). Ad-GFP expressing green fluorescence protein was used as a control for Ad infection. The cells were infected with Ad- N/ F Nox4 and Ad-GFP essentially as described previously [7,35]. The expression of human dominant-negative Nox4 was determined by RT–PCR analysis of total RNA isolated from infected 2T3 mouse cells using human Nox4-specific primers [34]. The expression of WT Nox4 was determined using immunoblot analysis as described previously [36]. In separate experiments, the cells were infected with Ad vector expressing catalase (Ad-catalase) (purchased from the University of Iowa Gene Transfer Vector Core, Iowa City, IA, U.S.A.). Catalase expression was detected using an antibody from Research Diagnostics by immunoblotting, as described previously [37].

Preparation of BMP-2-null osteoblasts from BMP-2 flx /flx mice

All animal experiments were performed according to IACUC guidelines as approved by the University of Texas Health Science Center at San Antonio.

BMP-2-floxed mice were generated as described previously [38]. Briefly, mice containing exon 3 of the *Bmp2* gene flanked by *Cre*-recombinase recognition sites (*loxP*) were generated to facilitate removal of the BMP-2 protein coding region upon expression of *Cre* recombinase. Primary osteoblast cells were isolated by sequential digestion with trypsin and collagenase [32,39]. These cells were infected with adenoviral vectors expressing *Cre* recombinase (Ad-*Cre*). Control infections were performed using Ad-GFP.

RNA extraction and RT-PCR analysis

Total RNA was isolated with TRI Reagent followed by chloroform extraction and precipitation of RNA with isopropyl alcohol. Total RNA (2 μ g) was reverse-transcribed to make cDNA in a 20 μ l reaction volume. After RT, 1 μ l of cDNA was amplified by quantitative PCR in a 25 μ l reaction volume in 96-well plates, using the ABI Prism 7900 sequence detection system and analysed by SDS 2.1 Software and a SYBR green probe method (Applied Biosystems). The PCR conditions were as follows: initial denaturation at 94°C for 10 min followed by 40 cycles at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. The fluorescence of the double-stranded products accumulated was monitored in real time. PCR reactions were performed in triplicate for each cDNA, averaged and the relative mRNA levels were normalized to the reference housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) at the same time in the same sample. A one-step RT-PCR kit was used to detect the expression levels of Nox4, Nox2, Nox1 using 100 ng of total RNA and a PCR cycling program as follows: 50°C for 20 min followed by initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s, annealing at 57°C for 30 s and extension at 72°C for 1 min. The primer sequences were: Nox1 forward, 5'-CTTCCTCACTGGCTGGGATA-3' and Nox 1 reverse, 5'-TGACAGCATTTGCGCAGGCT-3' (product length 219 bp); Nox2 forward, 5'-CCAGTGAAGATGTGTTTCAGCT-3' and Nox2 reverse, 5'-GCACAGCCAGTAGAAGTAGAT-3' (product length 155 bp); Nox4 forward, 5'-GAAGCCCATTTGAGGAGTCA-3' and Nox4 reverse, 5'-GGGTCCACAGCAGAAACTC-3' (product length 407 bp); human Nox4 forward, 5'-TCTCAGTGAATTACAGT-3' and human Nox4 reverse, 5'-AATGATGGTGACTGGC-3' (product length 550 bp); BMP-2 forward, 5'-TGAGGATTAGCAGGTCTTTG-3' and BMP-2 reverse, 5'-CACAACCATGTCCTGATAAT-3' (product length 440 bp); BMP-4 forward, 5'-ATTGGCTCCCAAGAATCATGG-3' and BMP-4 reverse, 5'-CGTGATGGAACTCCTCACAGT-3' (product length 427 bp); and BMP-7 forward, 5'-CGATTTTCAGCCTGGACAACG-3' and BMP-7 reverse, 5'-CCTGGGTACTGAAGACGG-3' (product length 254 bp). GAPDH primers were purchased from Superarray Biosciences.

ALP (alkaline phosphatase) staining and activity

Cells were seeded in 24-well plates at a density of 75000 cells per well and were allowed to grow to 90% confluency. For these experiments, the growth medium was supplemented with fresh ascorbic acid (100 mg/ml) and 2-glycerophosphate (5 mM). Cells were pretreated with either DPI, NAC or infected with Ad- N/ F Nox4 or Ad-catalase before addition of BMP-2 for 48 h. The cells were fixed in 10% formalin and stained using BCIP (5-bromo-4-chloroindol-3-yl phosphate) and NBT (Nitro Blue Tetrazolium) essentially as described previously [14,35]. The stained structures were examined and photomicrographed with a camera attached to the microscope. For the ALP assay, the treated cells were lysed using 0.05% Triton X-100 by repeated freeze-thawing and the clear lysates was assayed for ALP activity using PNPP (*p*-nitrophenyl phosphate) as the substrate, essentially as described previously [14,39]. ALP activity was normalized against the total protein amount and was

expressed as nmol/mg of protein per min. The data were from a representative of at least three experiments shown as the means $\pm$ S.E.M. of triplicate wells.

Mineralized bone nodule formation

Cells were plated in 24-well tissue culture plates ($n=3$ per group, 75000 cells/well) for 2 days until they reached confluency in a medium containing α MEM with 10% FBS. Then the growth medium was supplemented with differentiation medium containing serum-free α MEM, 7% FBS, 100 μ g/ml ascorbic acid and 5 mM 2-glycerophosphate before addition of BMP-2. The cells were cultured for an additional 10 days while the culture medium was changed (including test factors) every 2 days. Thereafter, the cultures were rinsed with PBS and fixed in ice-cold 70% ethyl alcohol for 1 h at 4°C. Subsequently cells were washed with distilled water and stained for 5 min with a 2% solution of Alizarin Red S (pH 4.0) for calcium detection. Nonspecifically bound stain was removed by washing with distilled water. Plates were dried and photomicrographed with a camera attached to the microscope. The stains were extracted with DMSO and for quantification, the absorbance was measured at 590 nm.

DCF (2',7'-dichlorofluorescein) assay to detect intracellular ROS

Peroxide-sensitive DCFH-DA is a cell-permeable dye. Intracellular esterases convert DCFH-DA into DCFH and ROS oxidizes it to highly fluorescent DCFH-DA. Cells were cultured in chamber slides. Serum-deprived cells were washed with HBSS (Hanks balanced salt solution), loaded with 10 μ M DCFH-DA and incubated for 30 min at 37°C. BMP-2 was added for the time periods indicated and differential interference contrast images were obtained using an Olympus Fluoview 500 confocal laser microscope [37,40].

NAD(P)H oxidase assay

NAD(P)H oxidase activity was determined by the lucigenin-enhanced chemiluminescence method as described previously [40,41]. Briefly, cells were rinsed five times in ice-cold PBS and scraped from the plate in PBS. The cell pellets were centrifuged at 800 g at 4°C for 10 min and were resuspended in lysis buffer [20 mM KH_2PO_4 (pH 7.0), 1 mM EGTA, 1 mM PMSF and 0.1% protease inhibitor cocktail]. Cell suspensions were homogenized with 100 strokes in a Dounce homogenizer on ice. To initiate the assay, 100 μ l of homogenate was added into 900 μ l of 50 mM phosphate buffer (pH 7.0), containing 1 mM EGTA, 150 mM sucrose, 5 μ M lucigenin as the electron acceptor and 100 μ M NADPH as an electron donor. Photon emission in terms of relative light units was measured every 30 s for 10 min in a luminometer. Superoxide production was expressed as relative chemiluminescence (light) units and was normalized by total protein.

Immunoblotting

Cells were washed with PBS and lysed using radio-immunoprecipitation buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 0.1% protease inhibitor cocktail and 1% Nonidet P40]. Extracts were left on ice for 30 min and the cell lysates were centrifuged at 16000 g for 15 min at 4°C to remove cell debris. Clear supernatant was collected and the protein concentration was determined using

the Bio-Rad protein assay using BSA as a standard. Equal amounts of protein (20 μ g) were separated by SDS/PAGE and transferred on to PVDF membrane and immunoblotted with the antibodies indicated as described previously [7,14,35,42].

Luciferase activity

2T3 cells stably expressing luciferase protein (2T3–Luc cells) were used to determine BMP-2-induced *BMP2* gene transcription [32,33]. Luciferase activity was determined in the cell lysates using a luciferase assay kit. The data were plotted as mean luciferase activity/ μ g of protein as arbitrary units $\pm$ S.E.M. [7,15,35,43].

Statistics

The significance of the data was determined by ANOVA followed by Student–Newman–Keuls analysis as described previously [15]. A *P* value less than 0.05 was considered as significant.

RESULTS

ROS generation is essential for BMP-2-induced osteoblast differentiation

BMP-2 promotes differentiation of osteoblast progenitors into mature osteoblasts. The expression of ALP by osteoblasts marks the initiation of osteoblast differentiation. We tested the role of ROS in ALP expression using 2T3 mouse pre-osteoblast cells [15,32]. We used NAC as an antioxidant. We stained the cells for ALP activity as a measure of expression of this enzyme. As expected, BMP-2 increased the expression of ALP (Figure 1A, compare b with a). Incubation of 2T3 cells with NAC inhibited BMP-2-induced ALP levels (Figure 1A, compare d with b). To confirm this observation, in a parallel experiment, we assayed the ALP activity in lysates of cells treated with NAC in the presence of BMP-2. NAC significantly blocked BMP-2-stimulated ALP activity *in vitro* (Figure 1B). These results indicate that BMP-2-induced ROS may regulate the early osteoblastic marker, ALP expression.

The physiological ROS are mainly produced by NAD(P)H oxidases [19]. We used DPI, an inhibitor of flavoprotein enzymes including NAD(P)H oxidases. Treatment of 2T3 cells with DPI inhibited BMP-2-induced ALP expression (Figure 1C, compare d with b). Similarly, DPI significantly prevented the increase in ALP activity in the cell lysates *in vitro* in response to BMP-2 (Figure 1D). In contrast, treatment of the cells with the mitochondrial respiratory chain complex I inhibitor rotenone had no effect on BMP-2-induced osteoblastic ALP enzyme activity (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/433/bj4330393add.htm>). Furthermore, expression of Ad-mediated catalase in these cells (Figure 1E) blocked BMP-2-induced ALP expression and enzyme activity (Figures 1F and 1G), indicating involvement of hydrogen peroxide in BMP-2-induced expression of ALP.

BMP-2 initiates osteoblast differentiation, leading to formation of mature osteoblasts. Fully differentiated osteoblasts interact with calcium phosphate, along with other organic and inorganic compounds, in response to various hormones and growth factors *in vivo* to form mineralized bone nodules [32]. We examined the role of ROS in this final stage of

osteoblastic differentiation *in vitro* using a mineralization assay followed by staining of the mineralized bone nodules with Alizarin Red S reagent. As expected, BMP-2 increased formation of mineralized nodule formation (Figure 2A, compare b with a). Antioxidant NAC abrogated BMP-2-induced nodule formation (Figure 2A, compare d with b). The extent of bone nodule formation was determined by measuring the Alizarin Red stain retained by the mineralized bone nodule. The quantification showed significant inhibition of BMP-2-induced mineralized bone formation (Figure 2B). Similarly, the NAD(P)H oxidase inhibitor prevented the formation of bone nodules in response to BMP-2 (Figure 2C, compare d with b, and Figure 2D). These results indicate that ROS regulate BMP-2-induced osteoblast differentiation and mature bone nodule formation.

BMP-2 activates ROS production in osteoblast cells

Many growth factors and cytokines increase ROS [19]. To evaluate the role of BMP-2 in initiating signals leading to ROS production in 2T3 pre-osteoblasts, we examined the generation of intracellular ROS with a fluorescence-based assay using the peroxide-sensitive fluorophore DCFH-DA, which mainly detects hydrogen peroxide produced by the dismutation of superoxide. Incubation of 2T3 cells with BMP-2 rapidly increased production of ROS in a time-dependent manner (Figure 3A). Treatment of cells with NAC blocked BMP-2-induced ROS generation (Figure 3B, compare d with b). Similarly, the NAD(P)H oxidase inhibitor DPI, as well as expression of catalase, inhibited production of ROS in response to BMP-2 in these cells (Figures 3C and 3D respectively, compare d with b). To examine the direct involvement of NAD(P)H oxidase in BMP-2-induced signal transduction, we tested superoxide production in 2T3 cells by measuring lucigenin-enhanced chemiluminescence using NAD(P)H as the substrate. Figure 3(D) shows a time-dependent increase in superoxide production in the lysates prepared from BMP-2-treated 2T3 cells. When this assay was performed using lysates from DPI plus BMP-2-treated cells, the superoxide production was significantly reduced (Figure 3E). These results demonstrate that BMP-2 produces ROS via NAD(P)H oxidase in these cells.

Nox4 regulates ALP activity in 2T3 cells

Multiple isoforms of NAD(P)H oxidases produce ROS. Tissue-specific expression of Nox isoenzymes have been reported [17]. To determine the expression of Nox isoforms in 2T3 cells, we performed RT-PCR analysis of the total RNA. Figure 4(A) shows that Nox4 is abundantly expressed in 2T3 cells; however, minimal expression of *Nox1* mRNA was also detected (lanes 4 and 2 respectively). No detectable level of Nox2 was identified (Figure 4A, lane 3). These results indicate that 2T3 cells predominantly express the Nox4 isoenzyme. Nox4 protein was also detected in 2T3 cells by immunoblotting using an anti-Nox4 antibody [36] (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/433/bj4330393add.htm>).

To investigate the role of Nox4 in osteoblast differentiation, we used an Ad vector expressing a dominant-negative Nox4. Wild-type Nox4 contains binding domains for two cofactors, NAD(P)H and FAD. The Ad contains human Nox4 with deletion of both the NAD(P)H- and FAD-binding domains (N/F), thus it confers dominant-negative activity when expressed in cells [34]. Using human Nox4-specific primers, it is possible to detect the expression of dominant-negative Nox4 [34]. Infection of 2T3 cells with this Ad vector

showed significant expression of N/ F *Nox4* mRNA within 24 h (Figure 4B). Next, we determined the involvement of Nox4 in BMP-2-induced ALP activity, which represents an early marker for osteoblast differentiation. As expected, BMP-2 increased ALP activity in these cells (Figure 4C). Ad-mediated expression of N/ F *Nox4* significantly inhibited BMP-2-stimulated ALP activity (Figures 4C). To directly determine the effect of Nox4, we used an Ad vector expressing wild-type enzyme. Expression of wild-type Nox4 significantly increased ALP activity in 2T3 cells (Figure 4D). These results demonstrate, for the first time, a role of Nox4 in ALP expression during osteoblast differentiation.

Nox4 regulates BMP-2-induced BMP-2 expression

We have previously reported that BMP-2 increases the expression of *BMP2* mRNA [6,32]. We confirmed this observation (Supplementary Figure S3A at <http://www.BiochemJ.org/bj/433/bj4330393add.htm>). Treatment of 2T3 cells, however, with BMP-2 did not increase the expression of other osteogenic BMPs, such as BMP-4 and BMP-7 (Supplementary Figures S3B and S3C). To confirm a specific role for BMP-2 in osteoblast differentiation, we used calvarial osteoblasts from a BMP-2-floxed mouse. Infection of these cells with an Ad vector expressing the *Cre* recombinase significantly blocked expression of *Bmp2* mRNA (Supplementary Figure S4A at <http://www.BiochemJ.org/bj/433/bj4330393add.htm>). As expected, incubation of BMP-2-floxed osteoblasts with recombinant BMP-2 increased the ALP activity (Supplementary Figure S4B). Expression of *Cre* recombinase, which attenuates *BMP2* mRNA expression, significantly prevented BMP-2-induced ALP activity (Supplementary Figure S4B). These results confirm a role for BMP-2-stimulated *BMP2* gene expression in osteoblast differentiation.

The signal transduction mechanism of BMP-2 expression is poorly understood. We investigated the involvement of ROS. Treatment of 2T3 cells with NAC or DPI significantly inhibited BMP-2-induced *BMP2* mRNA expression (Figures 5A and 5B). Similarly, expression of catalase prevented *BMP2* mRNA expression in response to BMP-2 (Figure 5C). Next, we examined the effect of dominant-negative Nox4 on *BMP2* mRNA expression. Expression of dominant-negative Nox4 significantly blocked BMP-2-stimulated expression of *BMP2* mRNA (Figure 5D).

To confirm our observation, we tested the role of ROS in regulating BMP-2 protein levels using immunoblotting. Incubation of 2T3 cells with BMP-2 for 24 h increased the abundance of BMP-2 in the lysates of PBS-washed cells (Supplementary Figure S5 at <http://www.BiochemJ.org/bj/433/bj4330393add.htm>, compare lane 4 with lane 3). To eliminate the possibility of detecting the recombinant BMP-2 added to the 2T3 cells, we incubated the cells with BMP-2 for 30 min followed by washing with PBS. Immunoblotting showed no detectable increase in levels of BMP-2 in these cell lysates (Supplementary Figure S5, compare lane 2 with lane 1). Treatment of 2T3 cells with both NAC and DPI followed by incubation with BMP-2 for 24 h significantly inhibited the expression of BMP-2 protein (Figures 6A and 6B, compare lane 4 with lane 2). Similarly, expression of catalase blocked BMP-2-induced BMP-2 protein levels in 2T3 pre-osteoblasts (Figure 6C, compare lane 4 with lane 2). Furthermore, expression of dominant-negative Nox4 markedly attenuated

expression of BMP-2 protein in response to BMP-2 (Figure 6D, compare lane 4 with lane 2). These results demonstrate that ROS, and specifically Nox4-derived ROS, regulate BMP-2 autoregulation of BMP-2 expression during osteoblast differentiation.

ROS regulate BMP-2 expression by transcriptional mechanisms

Previously, we showed that BMP-2 increases the transcription of the *BMP2* gene to maintain BMP-2 protein levels required for sustained differentiation of osteoblasts [6,14]. To test the role of ROS in the transcriptional regulation of BMP-2, we used 2T3 cells stably transfected with a reporter construct in which the luciferase cDNA is driven by mouse BMP-2 promoter (2T3–Luc cells) [32,33]. 2T3–Luc reporter cells maintained responsiveness to BMP-2-induced osteoblast differentiation [32]. These cells were treated with NAC or DPI or were infected with Ad-catalase to overexpress catalase protein, followed by incubation with BMP-2. BMP-2 treatment alone promoted transcription of the reporter gene, indicating increased *BMP2* gene transcription in response to BMP-2 (Figures 7A and 7B). Both NAC and DPI significantly inhibited BMP-2-induced transcription of BMP-2 (Figures 7A and 7B). Similarly, expression of catalase in 2T3–Luc cells markedly attenuated transcription of BMP-2 in response to recombinant BMP-2 (Figure 7C). Next, we tested the involvement of Nox4 in the transcription of BMP-2. As shown in Figure 7(D), Ad-mediated expression of dominant-negative Nox4 significantly blocked BMP-2-stimulated transcription of BMP-2. These results indicate that Nox4-derived ROS regulate *BMP2* gene transcription induced by BMP-2.

DISCUSSION

In the present paper, we report the induction of ROS generation in response to BMP-2 by activation of NAD(P)H oxidase. We demonstrate that ALP, the expression of which is required for osteoblast differentiation, requires production of ROS by BMP-2. Furthermore, our results provide the first evidence that the Nox4 isotype of NAD(P)H oxidase acts as a novel signalling molecule to produce physiological ROS, which modulate BMP-2 autoregulation necessary for osteoblast differentiation.

The generation of ROS by receptor–ligand interaction plays an important role in cell growth and differentiation [22,25,26]. Bone remodelling is a complex but extremely fine-tuned biological process requiring optimal activity of osteoblasts and osteoclasts. A role for ROS has been established for osteoclast differentiation, mainly in response to RANKL [29,30]. Previously, increasing the levels of ROS in osteoblasts, using hydrogen-peroxide- or xanthine-oxidase-generated superoxide anion, were reported to increase RANKL mRNA and protein expression [44]. Increased levels of ROS in hypertrophic chondrocytes were also shown to be associated with chondrogenic differentiation [45]. We have demonstrated that BMP-2 is a key player in co-ordinating osteoblast and osteoclast differentiation and has the potential to orchestrate bone remodelling [6,7,14]. In the present study we demonstrate that BMP-2 triggers ROS generation to induce ALP, resulting in initiation of osteoblast differentiation leading to formation of mature osteoblastic bone nodules (Figures 1 and 2).

Under physiological conditions, NAD(P)H oxidases, which produce superoxide anion, display very low constitutive activity [16,17]. Many growth factors such as angiotensin II,

insulin, PDGF (platelet-derived growth factor) and EGF (epidermal growth factor) produce hydrogen peroxide by activating the NAD(P)H oxidases [22,34,40,46]. Our results demonstrate activation of NAD(P)H oxidase by BMP-2 in the osteoblast progenitor cells, resulting in production of ROS, presumably in the form of hydrogen peroxide (Figure 3). The NAD(P)H oxidases represent isoforms of the founding member, neutrophil oxidase catalytic subunit gp91^{phox} (or Nox2) [16,17,47]. Nox4 shares overall structural similarity with Nox2 and is abundant in vascular tissues and kidney, especially in mesangial cells [16,17]. It was proposed that, in these tissues, Nox4-derived superoxide could lead to pathological conditions [48–50]. Our results demonstrate that Nox4 is also expressed abundantly in the pre-osteoblasts (Figure 4A). Furthermore using a dominant-negative enzyme, we show that Nox4 significantly contributes to the expression of the early osteoblastic marker ALP (Figure 4C), which is necessary for osteoblast differentiation. However, it should be emphasized that the effect of dominant-negative Nox4 expression is partial (Figure 4C). This observation indicates the involvement of other signalling pathways, which co-operate with Nox4 for the induction of osteoblast differentiation.

Superoxide anion generated by NAD(P)H oxidase is rapidly dismutated to hydrogen peroxide, which acts as the highly diffusible molecule to elicit physiological effects of ROS [19]. An established mechanism by which hydrogen peroxide regulates cell physiology involves inhibition of tyrosine phosphatases including the lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10), which contains catalytic cysteine susceptible to inactivation by hydrogen peroxide. However, in many protein tyrosine phosphatases, the hydrogen-peroxide-mediated oxidation to form disulfide linkage to nearby cysteine residues protects the catalytic cysteine from irreversible oxidation [51,52]. Although both BMPR I and BMPR II possess serine threonine kinase activity, induction of osteoblast differentiation by BMP-2 requires tyrosine phosphorylation of multiple proteins [14]. The mechanism by which BMP-2 increases this tyrosine phosphorylation is not understood. However, BMP-2-induced ROS generation and, in turn, inhibition of endogenous tyrosine phosphatases may represent a molecular mechanism of increased tyrosine phosphorylation, which leads to osteoblast differentiation [14]. This aspect of phosphatase regulation by ROS in response to BMP-2 during osteoblast differentiation requires further study.

We previously reported that the sustained action of BMP-2 on the formation of calcified bone nodules is maintained by its autoregulation [6,32]. The mechanism of BMP-2-induced BMP-2 expression is poorly understood. In the present study, we demonstrate that Nox4-derived ROS regulate *BMP2* mRNA expression (Figure 5). BMP-2-stimulated BMP-2 protein abundance is also modulated by ROS generated by Nox4 (Figure 6). Moreover, the present results demonstrate transcriptional regulation of the *BMP2* gene by ROS and Nox4 (Figure 7). Previously, we have reported that BMP-2-stimulated expression of BMP-2 protein is mediated by receptor-specific Smad5-dependent transcription [5,14]. Also, we showed a contribution of non-Smad PI3K/Akt signalling cascade to the Smad signalling for the regulation of BMP-2 transcription [14]. We now show a role for Nox4-derived ROS in transcriptional control of BMP-2 expression. Whether Nox4 contributes to the activation of PI3K and Smad5 signal transduction required for BMP-2 expression is under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Ad	adenovirus
ALP	alkaline phosphatase
BCIP	5-bromo-4-chloroindol-3-yl phosphate
BMP	bone morphogenetic protein
BMPR	BMP receptor
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DPI	diphenyleneiodonium
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
αMEM	α -minimal essential medium
NAC	N-acetyl-L-cysteine
NBT	Nitro Blue Tetrazolium
PI3K	phosphoinositide 3-kinase
PNPP	p-nitrophenyl phosphate
RANKL	receptor activator of NF- κ B (nuclear factor κ B) ligand
ROS	reactive oxygen species
RT	reverse transcription

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Figure 1. ROS regulate BMP-2-induced ALP expression

(A, C and F) Mouse 2T3 pre-osteoblasts were treated with 10 mM NAC (A) or 2.5 μ M DPI (C) for 1 h or were infected with Ad-catalase for 24 h (F) prior to incubation with 100 ng/ml BMP-2 for 48 h. The cells were incubated with the ALP substrate BCIP and NBT, and the stained cells were photomicrographed using a camera attached to microscope. (B, D and G) The cell lysates were assayed in vitro for ALP activity using PNPP as a substrate. Catalase expression was determined in the cell lysates by immunoblotting using an anti-catalase antibody (E, upper panel). An anti-actin antibody was used as a loading control (E, bottom panel). Values are means $\pm$ S.E.M. of triplicate measurements. * P < 0.001 compared with control; ** P < 0.001 compared with BMP-2-treated.

Figure 2. ROS regulate BMP-2-induced bone mineral formation

(**A** and **C**) 2T3 cells were treated with NAC (**A**) or DPI (**C**) as described in Figure 1. The cells were incubated with 100 ng/ml BMP-2 and stained with Alizarin Red S. Plates were photomicrographed using a camera attached to a microscope. (**B** and **D**) The stains in (**B**) and (**D**) respectively were extracted using DMSO and the absorbance was measured at 590 nm for quantification. Values are means $\pm$ S.E.M. of triplicate measurements. * P < 0.001 compared with control; ** P < 0.001 compared with BMP-2-treated.

Figure 3. BMP-2 increases ROS and NAD(P)H oxidase activity

(A) 2T3 cells were incubated with 100 ng/ml BMP-2 for the indicated periods of time. The production of ROS was determined by DCF fluorescence using a confocal laser-scanning fluorescence microscope. (B, C and D) 2T3 cells were treated with NAC (B) or DPI (C) for 1 h, or were infected with Ad-catalase for 24 h (D) followed by incubation with 100 ng/ml BMP-2 for 2.5 min. The production of ROS was determined by DCF fluorescence as described above. (E) 2T3 cells were incubated with 100 ng/ml BMP-2 for the indicated periods of time. The cell lysates were assayed for NAD(P)H oxidase activity using NAD(P)H and lucigenin. Superoxide generation was determined by photoemission every minute for 9 min and expressed as relative light units per μg of protein. (F) 2T3 cells were treated with 2.5 μM DPI for 1 h prior to incubation with 100 ng/ml BMP-2 for 2.5 min. The cell lysates were assayed for NAD(P)H oxidase activity as described in (E). Con, control.

Figure 4. Nox4 expression regulates BMP-2-stimulated ALP activity

(A) Total RNA isolated from 2T3 cells was used in RT-PCR with Nox1- (lane 2), Nox2- (lane 3) and Nox4- (lane 4) specific primers. The bottom panel shows amplification of GAPDH to demonstrate equal loading. Sizes of the PCR products are shown on the right in bp. 'M' indicates the 100 bp ladder. (B) Expression of dominant-negative N/ F Nox4. 2T3 cells were infected with Ad- N/ F Nox4 for 24 h. Ad-GFP was used as a control. Total RNA was isolated and used in RT-PCR analysis to detect human Nox4 using specific forward and reverse primers. (C) Expression of dominant-negative Nox4 inhibits BMP-2-induced ALP activity. 2T3 cells were infected with Ad- N/ F Nox4 for 24 h prior to incubation with 100 ng/ml BMP-2 as described in the legends to Figure 1(B). ALP activity was determined in the cell lysates as described in Figure 1. (D) Wild-type Nox4 expression increases ALP activity in osteoblasts. 2T3 cells were infected with Ad-Nox4 (wild-type) for 48 h followed by determination of ALP activity in cell lysates as described above. Values are means $\pm$ S.E.M. of triplicate measurements. * P < 0.001 compared with control; ** P < 0.05 compared with BMP-2-treated.

Figure 5. ROS regulate BMP-2-induced BMP2 mRNA expression

(A, B and C) 2T3 cells were treated with 10 mM NAC (A) and 2.5 μ M DPI (B) for 1 h prior to incubation with BMP-2 for 24 h. Cells were infected with Ad-catalase for 24 h followed by BMP-2 treatment for an additional 24 h (C). Total RNA was isolated and used in quantitative real-time RT-PCR using BMP-2-specific forward and reverse primers. (D) Expression of dominant-negative Nox4 inhibits BMP2 mRNA expression. 2T3 cells were infected with Ad- N/ F Nox4 for 24 h prior to incubation with 100 ng/ml BMP-2 as described above. Total RNA was used to detect BMP2 mRNA as described above. * P < 0.01 compared with control; ** P < 0.05 compared with BMP-2-treated (A); ** P < 0.01 compared with BMP-2-treated (B); ** P < 0.001 compared with BMP-2-treated (C) * P < 0.01 compared with control, ** P < 0.01 compared with BMP-2-treated (D).

Figure 6. ROS regulate BMP-2 protein expression

(**A**, **B** and **C**) 2T3 cells were treated with 10 mM NAC (**A**), 2.5 μ M DPI (**B**) or were infected with Ad-catalase (**C**), prior to incubation with BMP-2 as described for Figures 5(A)–5(C). The cell lysates were immunoblotted with anti-BMP-2, anti-catalase or anti-actin antibodies as indicated. (**D**) 2T3 cells were infected with Ad- N/ F Nox4 followed by BMP-2 as described for Figure 5(D). Cell lysates were immunoblotted with anti-BMP-2 and anti-actin antibodies as indicated. Parallel dishes of 2T3 cells were infected with Ad- N/ F Nox4 and incubated with BMP-2 in the same way. Total RNAs isolated from these cells were tested for human Nox4 expression to demonstrate expression of dominant-negative Nox4 and GAPDH as shown in the bottom two panels.

Figure 7. ROS regulate BMP2 gene transcription

(A, B and C) 2T3–Luc reporter cells were treated with 10 mM NAC (A), 2.5 μ M DPI (B) or were infected with Ad-catalase (C), followed by incubation with 100 ng/ml BMP-2 for 24 h. Luciferase activity was determined in the cell lysates and the protein was estimated using a luciferase assay kit. Values are means $\pm$ S.E.M. of triplicate measurements. * P < 0.001 compared with control; ** P < 0.001 compared with BMP-2-treated.

(D) Dominant-negative Nox4 inhibits BMP-2-induced BMP-2 transcription. 2T3–Luc cells were infected with Ad-N/ F for 24 h followed by incubation with 100 ng/ml BMP-2 for 24 h. Luciferase activity was determined in the cell lysates and the protein was estimated as described above. Values

are means $\pm$ S.E.M. of triplicate measurements. * P < 0.001 compared with control; ** P < 0.01 compared with BMP-2-treated.