Regulation of BMP2-Induced Intracellular Calcium Increases in Osteoblasts

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ABSTRACT: Although bone morphogenetic protein-2 (BMP2) is a well-characterized regulator that stimulates osteoblast differentiation, little is known about how it regulates intracellular Ca2+ signaling. In this study, intracellular Ca2+ concentration ([Ca2+]i) upon BMP2 application, focal adhesion kinase (FAK) and Src activities were measured in the MC3T3-E1 osteoblast cell line using fluorescence resonance energy transfer-based biosensors. Increase in [Ca2+]i, FAK, and Src activities were observed during BMP2 stimulation. The removal of extracellular calcium, the application of membrane channel inhibitors streptomycin or nifedipine, the FAK inhibitor PF-573228 (PF228), and the alkaline phosphatase (ALP) siRNA all blocked the BMP2-stimulated [Ca2+]i increase, while the Src inhibitor PP1 did not. In contrast, a gentle decrease of endoplasmic reticulum calcium concentration was found after BMP2 stimulation, which could be blocked by both streptomycin and PP1. Further experiments revealed that BMP2-induced FAK activation could not be inhibited by PP1, ALP siRNA or the calcium channel inhibitor nifedipine. PF228, but not PP1 or calcium channel inhibitors, suppressed ALP elevation resulting from BMP2 stimulation. Therefore, our results suggest that BMP2 can increase [Ca2+]i through extracellular calcium influx regulated by FAK and ALP and can deplete ER calcium through Src signaling simultaneously. © 2016 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 34:1725–1733, 2016.

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Bone morphogenetic protein-2 (BMP2) is a well-characterized regulatory factor in osteoblast cells that can induce osteoblast differentiation and bone formation1 by activating the intracellular signaling molecules such as Smad2, Runx2, and osterix (Osx/Sp7).3 It has also shown that BMP2 positively regulates the expression of integrins and PI3K/Akt signaling cascade to further promote osteogenic differentiation.4,5 These studies exemplify the wide variety of crosstalk that occurs among signal transduction cascades during the differentiation, which is represented by the extracellular calcium deposition. However, intracellular Ca2+, which is not the source of calcium deposition, can also modulate numerous physiological responses as a second messenger6 and acts as a co-factor for the differentiation.7 Thus, the objective of this study is to explore the potential pathways of intracellular Ca2+ signal that participate in BMP2-induced osteoblast differentiation.

In this study, the dynamics of intracellular Ca2+ concentration ([Ca2+]i) was observed using fluorescence resonance energy transfer (FRET) technology; the underlying molecular basis was explored through examining the activation of focal adhesion kinase (FAK), Src, and alkaline phosphatase (ALP). The results of this study indicate that BMP2 promotes an increase in [Ca2+]i, mediated by FAK, Src, and ALP through the extracellular calcium influx and subsequent endoplasmic reticulum (ER)-stored calcium release.

MATERIALS AND METHODS

Cell Culture

MC3T3-E1 osteoblast cell line obtained from the American Type Culture Collection (ATCC) was cultured in a humidified incubator with high glucose Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Invitrogen, Grand Island, NY) and 5% CO2 at 37°C. The DMEM contained 10% fetal bovine serum (FBS, Invitrogen), 2 mmol/L L-glutamine, 100 unit/ml penicillin, and 100 μg/ml sodium pyruvate.

Gene Construction and DNA Plasmids

A genetically encoded FRET biosensor based on the enhanced cyan fluorescent protein (ECFP) and yellow fluorescent protein for energy transfer (YPet) (Cyto-Ca2+) was used to monitor the intracellular calcium concentration as previously described in the literature.8 An ER-targeted calcium biosensor (ER-Ca2+) was used to detect the ER-stored calcium concentration.9 A lyn-targeted FAK biosensor10 and a Kirsten rat sarcoma viral oncogene homolog (Kras)-targeted Sre FRET biosensor (Kras-Src) were used to monitor the FAK and Src activity, respectively. The emission ratio of ECFP/YPet from the Cyto-Ca2+, FAK, and Src FRET biosensors represents Cyto-Ca2+ concentration and enzymatic activity, respectively, while the emission ratio of YPet/ECFP represents the ER-Ca2+ concentration.9 All the DNA plasmids for the FRET biosensors are pcDNA3.1(+), and these FRET biosensors were transfected into MC3T3-E1 cells 48 h prior to the stimulation experiment using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, respectively.

Wenfeng Xu and Bo Liu contributed equally to this work.

Conflicts of interest: None.

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ALP Activity Assay
MC3T3-E1 cells were harvested, followed by cell lysis with TritonX-100. The ALP activities were quantified by a detection kit obtained from Santa Cruz Biotechnology, Dallas, TX. The examination was performed with the ALP reagent set and UV spectrophotometer (measured at 405 nm). The quantified results were normalized by the protein concentration. 3.3 nmol ALP-siRNA (Dharmacon, Laffayette, CO) was dissolved in 330 μl RNAse-free distilled water and then stored at -20°C, and 100 nmol/L of ALP-siRNA was transfected into the cells using Lipofectamine 2000 (Invitrogen).

Drugs and Chemicals
All the chemicals and reagents used in this study, unless otherwise noted, were obtained from Sigma–Aldrich (St. Louis, MO). Nifedipine (NIF) and 2-Aminoethyl diphenylborinitate (2-APB) were prepared as stock solutions in DMSO, frozen at –20°C and thawed immediately before experiments. For experiment using inhibitors, the cells were pre-treated for 1 h with voltage-operated calcium channel (VOCC) inhibitor NIF (10 μmol/L), stretch-activated calcium channel (SACC) inhibitor streptomycin (STM, 200 μmol/L), or intracellular IP3-induced calcium release inhibitor (2-APB, 100 μmol/L), respectively, before FRET imaging experiment. In addition, the pre-treatment of FAK inhibitor PF-573228 (PF228, 20 μmol/L), Src inhibitor PP1 (20 μmol/L), or calcium free DMEM were 30 min, respectively.

Microscope, Image Acquisition, and Analysis
MC3T3-E1 cells expressing transfected FRET biosensors were starved using 0.5% FBS in DMEM for 24 h before imaging experiments. All images were obtained from isolated single cells under an inverted microscope (Nikon Eclipse Ti Series, Ti-Epi-fl/1, Nikon, Tokyo, Japan) equipped with a cold charge-coupled device (CCD) (EVO-512-M-FW-16-AC, Photometrics, Tucson, AZ), a 440DF20 excitation filter, a 455DRLP dichroic mirror, and two emission filters with controllers (480DF30 for CFP and 535DF25 for YFP). Time-lapse fluorescence images were acquired and quantified by MetaFluor 7.0 software (Universal Imaging, Downingtown, PA), and the FRET ratio data were analysis by Excel (Microsoft, Redmond, WA).

Statistical Analysis
Five to fifteen cells were tested on each culture dish, and all cell data on one dish were averaged as one sample. In this paper, “n” means the number of dishes. All data were normalized to a basal level before stimulation of a cell. The error bars in the line charts and histograms show standard deviations (SD). In the line charts, data from point before stimulation (0 s) and the last point (440 s) were picked up for histogram plot. Statistical analysis was performed using Student’s t-test function in Excel (Microsoft) to evaluate any significant differences between two groups. A p-value < 0.05 was considered significant. A post-hoc test (Bonferroni) was performed using MATLAB software (MathWorks, Novi, MI) when there were more than two groups to confirm the conclusions.

RESULTS
BMP2 Stimulates an Increase in Intracellular Calcium
First, the spatiotemporal characteristic of [Ca2+]i under the BMP2 stimulation was examined since the alternation of calcium concentration is essential to the function of osteoblast cell. Figure 1A and B present that [Ca2+]i in MC3T3-E1 cells gradually increased 40% above baseline after 10 min of treatment with 2 μM BMP2. An obvious [Ca2+]i increase was also observed upon 2 μM BMP2 stimulation (but not phosphate buffered saline (PBS)) when the calcium indicator Fluo-4 was used to stain the intracellular calcium (Supplemental Fig. S1). This confirmed the result of BMP2-induced [Ca2+]i increase using FRET biosensor Cyto-Ca2+. The result in Figure 1A and C indicates a 20% decrease in ER calcium within 10 min of the same BMP2 treatment.

To identify the source of the BMP2-induced [Ca2+]i increase, MC3T3-E1 cells were cultured with calcium free DMEM for 30 min. Thus, prior to the BMP2 stimulation, the removal of extracellular calcium was achieved; and the change in both the cytosolic and ER calcium concentrations disappeared. This result shown in Figure 1A and B demonstrates that the extracellular calcium is necessary for the BMP2-induced increase in [Ca2+]i observed in MC3T3-E1 cells. To further test the contribution of intracellular calcium store, 2-APB, an IP3-induced ER calcium release inhibitor, was pre-treated for 1 h before imaging. As a result shown in Figure 1A and B, 100 μmol/L of 2-APB attenuated the BMP2-induced [Ca2+]i increase to 12%. A smaller decrease of 6% in ER calcium concentration was observed during the application of BMP2 (shown in Fig. 1A and C). These results indicate that the ER calcium release is also involved in the BMP2-induced increase of [Ca2+]i.

Different calcium channels allow calcium influx across the plasma membrane. To further identify which channels contribute to the BMP2-induced increase in [Ca2+]i, MC3T3-E1 cells transfected with the cytosolic calcium biosensor were pre-treated with the inhibitor of SACC STM13 or the inhibitor of VOCC NIF for 1 h. The result in Figure 2A and B indicates that pre-treatment of cell with 200 μmol/L STM or 10 μmol/L NIF significantly inhibited the BMP2-induced increase in [Ca2+]i. This suggests that both SACC and VOCC may be of significance.

MC3T3-E1 cells expressing the ER calcium biosensor were then pre-treated with STM or NIF. In this case, the effect of NIF on the BMP2-induced calcium decrease inside the ER is insignificant (shown in Fig. 2C and D). In contrast, STM appeared to inhibit the decrease. These results suggest that the increase in [Ca2+]i, in response to BMP2 treatment is dependent upon both extracellular and intracellular calcium reservoirs, and the ER calcium release may be partly reliant on the influx of extracellular calcium.

BMP2 Activates FAK and Src
To explore whether BMP2 affects FAK activity, a FRET-based FAK biosensor was transfected into MC3T3-E1 cells. The result shown in Figure 3 presents an increase in the FRET ratio from the FAK biosensor after the BMP2 stimulation. This increase was
prevented by pre-treatment with the FAK inhibitor PF228 (20 μmol/L) for 30 min prior to the BMP2 treatment, showing that change in the FRET ratio from the FAK biosensor is from FAK activity in response to the stimulation. However, 30 min of the pre-treatment with Src inhibitor PP1 (20 μmol/L) had no effect on BMP2-induced FAK activation in MC3T3-E1 cells.

Src can form a complex with FAK and recruit it to regulate signaling cascades and cellular functions; thus, in this study, BMP2-induced Src activity was also examined using a membrane-bound Src FRET biosensor (Kras-Src). The result shown in Figure 4 expresses that Src activity was significantly elevated in response to the treatment with BMP2 but was totally inhibited to the treatment with PP1. In addition, the pre-treatment of cells with PF228 also prevented BMP2-induced Src activation, inferring that BMP2 alters Src activity through FAK.

**FAK and Src Play Roles in BMP2-Induced [Ca^{2+}]_{i} Increase**

To explore whether FAK or Src participates in increasing [Ca^{2+}]_{i} upon BMP2 treatment, MC3T3-E1 cells transfected with either the Cyto-Ca^{2+} or ER-Ca^{2+} biosensors were pre-treated with PF228 or PP1 prior to the BMP2 stimulation. The BMP2-induced increase in [Ca^{2+}]_{i}, as measured by the Cyto-Ca^{2+} biosensor, was significantly inhibited by PF228 but not by PP1 (Fig. 5A and B). In contrast, the BMP2-induced ER calcium release was restrained significantly by PP1 but not PF228 (Fig. 5C and D). These results indicate that upon the stimulation, FAK can regulate the influx of extracellular calcium, while Src participates in the release of ER calcium.

Our subsequent studies were to pre-treat FAK and Src FRET biosensor transfected cells with STM or NIF for further exploration of the relationship between changes in [Ca^{2+}]_{i} and FAK/Src activity. The result shown in Figure 6 demonstrates that the blocking of SACC by STM did not inhibit BMP2-induced FAK or Src activation. In contrast, blocking of VOCC by NIF completely inhibited BMP2-induced FAK and Src activation. These results suggest that the BMP2-induced FAK/Src activation is mediated by normal VOCC function but is independent of SACC.

**ALP Participates in the BMP2-Induced Increase in [Ca^{2+}]_{i}**

The expression of ALP, due to the BMP2-induced increase in [Ca^{2+}]_{i}, was measured in this study since ALP plays an essential role in the bone formation of
Figure 2. The effects of calcium channel inhibitors on BMP2-induced calcium changes. Representative images of the emission ratio of Cyto-Ca\(^{2+}\) FRET biosensor (A) or ER-Ca\(^{2+}\) FRET biosensor (C) in MC3T3-E1 cells pre-treated with 200 \(\mu\)mol/L STM or 10 \(\mu\)mol/L NIF before BMP2 stimulation. The average time course of the (B) cytoplasmic Ca\(^{2+}\) concentration (\(n = 6\) for STM, \(n = 7\) for NIF) or (D) ER Ca\(^{2+}\) concentration pre-treated with STM (\(n = 5\)) or NIF (\(n = 7\)). Scale bars: 10 \(\mu\)m (A and C). The difference was significant between ER-Ca\(^{2+}\) + NIF and ER-Ca\(^{2+}\) + STM after 280 s (D) (\(p < 0.05\)).

Figure 3. BMP2-induced FAK activation was not affected by PP1. (A) Representative ratio images and (B) the time course of the FRET biosensor to show FAK activation before and after BMP2 stimulation in MC3T3-E1 cells pre-treated with (\(n = 6\)) or without 20 \(\mu\)mol/L PF228 (\(n = 6\)) or 20 \(\mu\)mol/L PP1 (\(n = 6\)) for 30 min (C). Bar graphs represent the change in the average value of emission ratios measuring FAK activation with or without BMP2 stimulation pre-treated with PP1 or PF228. Scale bars: 10 \(\mu\)m (A). The difference was significant between FAK and FAK + PF228 after 240 s (B) (\(p < 0.05\)). *\(p < 0.05\) (C).
Figure 4. BMP2-induced Src activation is mediated by FAK. (A) Representative images and (B) time course of Kras-Src FRET biosensor to show Src activation before and after BMP2 stimulation in MC3T3-E1 cells pre-treated with ($n = 7$) or without $20 \mu$mol/L PP1 ($n = 6$) or $20 \mu$mol/L PF228 ($n = 6$) for 30 min. (C) Bar graphs represent the change in the average value of emission ratios measuring Src activation with or without BMP2 stimulation pre-treated with PP1 or PF228. Scale bars: 10 $\mu$m (A). The differences were significant between Src and Src + PF228 or Src and Src + PP1 after 160 s (B) ($p < 0.05$). $C_3 p < 0.05$ (C).

Figure 5. BMP2 induces calcium changes in cells pre-treated with PF228 or PP1. Representative emission ratio images of Cyto-Ca$^{2+}$ FRET biosensor (A) or ER-Ca$^{2+}$ FRET biosensor (C) in MC3T3-E1 cells pre-treated with $20 \mu$mol/L PP1 or $20 \mu$mol/L PF228 for 30 min before BMP2 stimulation. The average time course of the (B) cytoplasmic Ca$^{2+}$ concentration ($n = 7$ for PP1, $n = 8$ for PF228) or ER Ca$^{2+}$ concentration ($n = 5$ for PP1, $n = 6$ for PF228) pre-treated with PP1 or PF228. Scale bars: 10 $\mu$m (A and C). The differences were significant between Cyto-Ca$^{2+}$ + PF228 and Cyto-Ca$^{2+}$ + PP1 after 240 s (B) or between ER-Ca$^{2+}$ + PF228 and ER-Ca$^{2+}$ + PP1 after 200 s (D) ($p < 0.05$).
osteoblasts. After 2 min of BMP2 treatment, it was observed an approximately 60% increase in ALP expression compared to that of the control group. Pretreatment with the FAK inhibitor PF228 prior to the BMP2 stimulation significantly reduced ALP expression to 55% lower than the control. Treatment of cells with the Src inhibitor PP1 did not significantly inhibit BMP2-induced ALP expression compared to that of the control (Fig. 7A). The results show that the increase of ALP activity induced by BMP2 depends on FAK but not Src.

Subsequently, the ALP-siRNA was also transfected into those MC3T3-E1 cells containing different FRET biosensors to prevent ALP expression prior to the BMP2 stimulation. The efficiency of RNA interference is shown in Figure 7A. Upon BMP2 treatment, ALP-siRNA was found to block Src activation, the increase of $[\text{Ca}^{2+}]_i$, and the release of ER calcium significantly, while it did not prevent the FAK activation (shown in Fig. 7B and C). These results indicate that FAK regulates the BMP2-induced increase of $[\text{Ca}^{2+}]_i$ through ALP, and that ALP alters the release of calcium from ER through Src activation.

**DISCUSSION**

As an important second messenger molecule, $\text{Ca}^{2+}$ impacts nearly every aspect of cellular life, including osteoblast differentiation and bone formation. The cytosolic resources of $\text{Ca}^{2+}$ include extracellular influx and intracellular ER-stored $\text{Ca}^{2+}$ release. Both of them can be operated in osteoblasts in a spatiotemporal manner by physical factors, such as shear stress and physiologic electrical stimulation, or by chemicals such as Leukotriene B4 and the transforming growth factor-β (TGF-β). Although BMP2 has been well reported to promote osteoblast differentiation and bone formation, change in the intracellular $\text{Ca}^{2+}$ concentration in response to the BMP2 stimulation has not been elucidated. In the current study, FRET data from two types of $\text{Ca}^{2+}$ biosensors were used to show that BMP2 can significantly elevate the intracellular calcium concentration simultaneously through the influx from extracellular $\text{Ca}^{2+}$ and the release of extracellular $\text{Ca}^{2+}$-dependent ER calcium.

The clearest association of calcium channels with cellular function is the L-type voltage sensitive channels on the cell membrane of osteoblasts, which mediate $[\text{Ca}^{2+}]_i$ through many signals, such as cell stretch, protein receptors, vitamin D, or parathyroid hormone. The L-type calcium channels are reported to be the major source of intercellular calcium signals in osteoblasts. However, although L-type calcium channels are essential for mediating intercellular calcium levels, some pharmacological inhibitor studies of voltage sensitive channels have shown a variety of results in osteoblast differentiation. Indeed, the results from this study show a different mechanism of extracellular calcium influx upon BMP2 application.
The channels through which calcium enters in response to the BMP2 stimulation may not be only due to VOCC but also SACC since both nifedipine and streptomycin inhibited BMP2-induced changes in \([\text{Ca}^{2+}]_i\).

It has been reported that both the FAK antibody and the FAK lacking tyrosine kinase activity (a FAK related non-kinase, FRNK) can inhibit the \([\text{Ca}^{2+}]_i\) current through L-type channels,24 a major outside-in pathway mediated by integrins.25 In addition, a common intracellular Ca\textsuperscript{2+} compartment release mechanism is the activation of phospholipase C (PLC) to cleave phosphatidylinositol bisphosphate (PIP\textsubscript{2}) into inositol triphosphate (IP\textsubscript{3}) and diacylglycerol (DAG). Then, IP\textsubscript{3} binding to the IP\textsubscript{3} receptor (IP\textsubscript{3}R) on the ER allows the diffusion of Ca\textsuperscript{2+} from the ER to the cytosol.15 Reportedly, many molecules can activate PLC such as G-protein coupled receptors and Src;\textsuperscript{26} therefore, Src could potentially mediate BMP2 binding on the cell membrane and calcium release from the ER. This study has demonstrated the reported argument since BMP2 can activate Src and the Src inhibition can significantly suppress ER calcium release, which is similar to our previous report.\textsuperscript{27} The role of Src may be mediated by PLC activation, since it binds to the exposed docking region of PLC-\textgamma to phosphorylates and activates PLC-\textgamma,\textsuperscript{28} which further leads to IP\textsubscript{3} production and ER-stored calcium release.\textsuperscript{26}

Then why FAK and Src can participate in BMP2-induced calcium alternation? BMP2 has been reported to upregulate the expression of integrins, which play a critical role in BMP2's function in osteoblasts.\textsuperscript{29,30} Therefore, it is possible that BMP2 promotes FAK activation via integrins\textsuperscript{31,32} on the plasma membrane to activate the downstream signals that regulate osteoblast differentiation. Indeed, the FAK activity was elevated significantly during BMP2 treatment along with the upregulation of the osteoblast marker ALP. This result is consistent with a previous report.\textsuperscript{32} In addition, this study has also shown that Src can be activated by the BMP2 stimulation and is part of the downstream signaling cascade from the BMP2-induced FAK activation. This activation may be due to the integrin-mediated autophosphorylation of FAK at tyrosine 397, creating a binding site for the SH2 domain of Src.\textsuperscript{33} Although FAK and Src are known to form complexes and cooperate at focal adhesion sites to promote cell behaviors such as migration, proliferation, survival, and differentiation,\textsuperscript{10,34} this study, examining the mechanism of BMP2-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i}, demonstrates a separate regulation of FAK and Src on cytosolic Ca\textsuperscript{2+} resources, which is consistent with a previous report.\textsuperscript{35} The extracellular Ca\textsuperscript{2+} influx in response to BMP2 is regulated by FAK, while Src regulates ER-calcium release. This distinction maybe
due to different localizations (FAK activity resides mainly inside lipid rafts while Src activity is concentrated in non-raft regions at the plasma membrane\(^{10,35}\)) and may result in their distinct regulatory mechanisms during the differentiation process of osteoblasts.

As an osteoblast marker and sensitive target reflecting osteoblast activity, ALP transforms organophosphate to inorganic phosphorus in osteoblasts (needed for the mineralization of osteoblasts) and also promotes the dephosphorylation of corresponding substrates during the process of bone formation.\(^{35}\) The current study clearly shows upregulated expression of ALP upon the BMP2 stimulation, highlighting the essential role of ALP in osteoblasts differentiation. Interestingly, based on the observation that ALP siRNA inhibited the BMP2-induced increase in \([\text{Ca}^{2+}]_i\), BMP2-induced ALP expression also monitors intracellular calcium increase. It can be concluded that ALP lies downstream of FAK since the FAK inhibitor PF228 decreases ALP expression and ALP siRNA does not affect FAK activation after the stimulation.

In this study, although a clear signal transduction pathway has been figured out based on the finding, interestingly a reverse regulation of intracellular calcium on BMP2-induced ALP expression (Supplemental Fig. S2) and Src/FAK activation was also found. The finding demonstrates a regulatory network among these signal factors, which should be a common phenomenon within the calcium related signal transduction.\(^{36,37}\) Further exploration is still needed to identify the molecular mechanism and the relationship among FAK, Src, and ALP in BMP2-mediated changes in calcium concentration during osteoblasts differentiation.

**AUTHORS’ CONTRIBUTIONS**

B. Liu and X. Liao designed the research project; W.X., X. Liao and X. Liu performed the research; X. Liao, B. Liu and X. Liu analyzed data; B. Li and Z.X. cultured cells; B. Liu, X. Liao, X. Liu; and M.C. wrote the paper. All authors have read and approved the final submitted manuscript.

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