Bone morphogenetic protein 4 enhances canonical transient receptor potential expression, store-operated Ca\(^{2+}\) entry, and basal [Ca\(^{2+}\)]\(_{i}\) in rat distal pulmonary arterial smooth muscle cells

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Lu W, Ran P, Zhang D, Lai N, Zhong N, Wang J. Bone morphogenetic protein 4 enhances canonical transient receptor potential expression, store-operated Ca\(^{2+}\) entry, and basal [Ca\(^{2+}\)]\(_{i}\) in rat distal pulmonary arterial smooth muscle cells. Am J Physiol Cell Physiol 299: C1370–C1378, 2010. First published September 15, 2010; doi:10.1152/ajpcell.00040.2010.—Recent advances have identified an important role of bone morphogenetic protein 4 (BMP4) in pulmonary vascular remodeling, yet the underlying mechanisms remain largely unexplored. We have previously found that Ca\(^{2+}\) influx through store-operated calcium channels (SOCC), which are mainly thought to be composed of canonical transient receptor potential (TRPC) proteins, likely contribute to the pathogenic development of chronic hypoxic pulmonary hypertension. In this study, we investigated the effect of BMP4 on expression of TRPC and store-operated Ca\(^{2+}\) entry (SOCE) in pulmonary arterial smooth muscle cells (PASMCs). Real-time quantitative PCR and Western blotting revealed that treatment with BMP4 (50 ng/ml, 60 h) increased TRPC1, TRPC4, and TRPC6 mRNA and protein expression in growth-arrested rat distal PASMCs. Moreover, in comparison to vehicle control, cells treated with BMP4 also exhibited enhanced SOCE, and elevated basal intracellular calcium concentration ([Ca\(^{2+}\)]\(_{i}\)) as determined by fluorescent microscopy using the Ca\(^{2+}\) indicator Fura-2 AM. Perfusing cells with Ca\(^{2+}\)-free Krebs-Ringer bicarbonate solution (KRBS) or KRBS containing SOCC antagonists SKF-96365 or NiCl\(_{2}\) attenuated the increases in basal [Ca\(^{2+}\)]\(_{i}\) caused by BMP4. Specific knockdown of BMP4 by small interference RNA significantly decreased the mRNA and protein expression of TRPC1, TRPC4, and TRPC6 and reduced SOCE and basal [Ca\(^{2+}\)]\(_{i}\) in serum-stimulated PASMCs. We conclude that BMP4 regulates calcium signaling in PASMCs likely via upregulation of TRPC expression, leading to enhanced SOCE and basal [Ca\(^{2+}\)]\(_{i}\) in PASMCs, and by this mechanism contributes to pulmonary vascular remodeling during pulmonary arterial hypertension.

calcium signaling; intracellular calcium concentration

Bone morphogenetic proteins (BMPs) are multifunctional growth factors of the transforming growth factor-β superfamily that regulate growth and differentiation in many cell types and play remarkable roles in embryonic development, organogenesis, and adult tissue remodeling (6, 25, 27). Members of the BMP family signal through the complex of type I (i.e., Alk2, Alk3, and Alk-6) and type II (i.e., BMPRII, ActRIIa, and ActRIIb) serine/threonine kinase receptors, leading to activation of Smad-dependent and Smad-independent (e.g., ERK, JNK, and p38 MAP kinase pathways) regulation of gene expression (6, 25). Recent evidence has identified multiple abnormalities in BMP signaling associated with pulmonary arterial hypertension (PAH), a lethal complication featured by profound remodeling and sustained vasoconstriction in pulmonary vasculature (9). For example, loss-of-function germline mutations of BMPRII were found in many cases of familial and sporadic PAH (5, 20, 24, 40). Marked reduction of BMPRII expression in pulmonary vasculature was found to be associated with primary pulmonary hypertension and in animal models of pulmonary hypertension induced by monocrotaline or chronic hypoxia (12, 29, 39, 10). Increased expression of BMP ligands (e.g., BMP2, BMP4, and BMP7) or downstream SMAD and p38\(^{\text{MAPK/ERK}}\) signaling was observed in hypoxic mouse lung (10). In the past decade, more and more effort has been devoted to understanding the role and mechanism of aberrant BMP signaling in the development of PAH.

It is generally accepted that elevation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) facilitates the contraction and proliferation of PASMCs and is therefore considered to be a key contributor to the pathogenesis of pulmonary hypertension (3, 26, 14). The increase in [Ca\(^{2+}\)]\(_{i}\) could result from the following (16, 36): 1) release of Ca\(^{2+}\) from internal storage sites, such as sarcoplasmic reticulum (SR); 2) influx of Ca\(^{2+}\) from extracellular fluid through L-type voltage-dependent Ca\(^{2+}\) channels (VDCC), receptor-operated Ca\(^{2+}\) channels, or SOCC; and/or 3) reduced efflux of Ca\(^{2+}\) through plasmalemmal Ca\(^{2+}\)-ATPases and Na\(^{+}\)/Ca\(^{2+}\) exchange. Ca\(^{2+}\) entry via SOCC, so-called store-operated Ca\(^{2+}\) entry (SOCE), is triggered by depletion of SR Ca\(^{2+}\) stores and is essential to refill Ca\(^{2+}\) in SR, to maintain intracellular Ca\(^{2+}\) homeostasis, and to elicit pulmonary vasostriction (30, 37, 34, 45). In pulmonary arterial smooth muscle cells (PASMCs), we and others have previously found that Ca\(^{2+}\) entry via SOCC other than VDCC provides an important pathway for hypoxic increases of [Ca\(^{2+}\)]\(_{i}\) (21, 23, 42–44). In response to growth factors, such as PDGF, proliferation of PASMCs occurs with increased SOCE and [Ca\(^{2+}\)]\(_{i}\) (11, 12, 38, 50). The molecular identity of SOCC is not completely understood. It is believed that SOCC are composed of a subgroup members of the transient receptor potential (TRP) superfamily, termed canonical transient receptor potential (TRPC) proteins (31). Of the seven TRPC (TRPC1–7) isoforms identified so far, we recently demonstrated that only TRPC1, TRPC4, and...
TRPC6 are substantially expressed in rat distal pulmonary artery smooth muscle and PASMCs (22, 41, 43).

BMP4, a member of the BMP family, was recently recognized as an important factor promoting PASMC proliferation and migration, and ultimately mediating pulmonary vascular remodeling in chronically hypoxic pulmonary hypertension (CHPH) (10); however, the detailed mechanisms remain unknown. In this study we examined the regulation of the calcium signaling by BMP4 in PASMCs. Our results indicate that BMP4 augments Ca$^{2+}$ influx through SOCC and elevates basal [Ca$^{2+}$], in PASMCs likely via induction of TRPC expression, thus providing a mechanism of its contribution to the pathogenesis of PAH.

MATERIALS AND METHODS

Isolation and culture of rat distal pulmonary arterial smooth muscle cells. Animal protocols were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions. Distal pulmonary arteries (PA, >4th generation) were dissected from lungs of male Wistar rats (300–500 g body wt) and anesthetized with pentobarbital sodium (65 mg/kg ip) as previously described (41). Adventitia were removed from the isolated PA, and endothelium was denuded by opening the vessel longitudinally and rubbing the luminal surface with a cotton swab. PASMCs were harvested from these vessels enzymatically and plated onto 25-mm coverslips in six-well dishes and cultured for 3–5 days in smooth muscle growth media (SMGM-2, Clonetics, Walkersville, MD) in a humidified atmosphere of 5% CO$_2$-95% air at 37°C. Cellular purity was >95%, as assessed by morphological appearance under phase-contrast microscopy and immunofluorescence staining for α-actin.

BMP4 treatment. PASMCs at 50–60% confluence were grown-stained smooth muscle basal media (SMBM, Clonetics, Walkersville, MD) containing 0.3% FBS for 24 h and then treated with 5–250 ng/ml recombinant human BMP4 (rhBMP4, R&D systems, Minneapolis, MN) for 24, 48, or 60 h, before they were subjected to Ca$^{2+}$ assays or gene expression measurements.

Small interference RNA transfection. BMP4 small interference RNA (siRNA) was designed and synthesized by Dharmacon (Lafayette, CO). It is a siGENOME SMARTpool containing four individual duplexes targeting to different areas of BMP4 mRNA (accession no. NM_012827). Nontargeting siRNA Pool (Dharmacon) served as a negative control. siRNA was designed and synthesized by Dharmacon (Lafayette, CO). It is a siGENOME SMARTpool containing four individual duplexes targeting to different areas of BMP4 mRNA (accession no. NM_012827). Nontargeting siRNA Pool (Dharmacon) served as a negative control.

RNA extraction and real-time quantitative PCR. Total RNA in PASMCs was extracted using RNeasy kit (Qiagen, Valencia, CA). DNA contamination in RNA preparations was removed by on-column DNase digestion using RNeasy column and RNase-free DNaseI (Qiagen). Transcript mRNA was designed and synthesized by Dharmacon (Lafayette, CO). It is a siGENOME SMARTpool containing four individual duplexes targeting to different areas of BMP4 mRNA (accession no. NM_012827). Nontargeting siRNA Pool (Dharmacon) served as a negative control.

As we described previously (41), cover-slips with PASMCs were loaded with 7.5 μM Fura-2 AM (Invitrogen) for 60 min at 37°C under 5% CO$_2$-95% air, and each was mounted in a closed polycarbonate chamber clamped in a heated aluminum platform (PH-2, Warner Instruments, Hamden, CT) on the stage of a Nikon TSE 100 Eclipse inverted microscope (Nikon, Melville, NY). Cells were perfused at 0.5–1 ml/min with Krebs-Ringer bicarbonate (KRB) solution (KRBBS), which was composed of (in mM) 118 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 0.57 MgSO$_4$, 1.18 KH$_2$PO$_4$, 25 NaHCO$_3$, and 10 glucose, equilibrated in heated reservoirs with 5% CO$_2$ and 16% O$_2$ and led to the chamber through stainless steel tubing. Chamber temperature was maintained at 37°C with an in-line heat exchanger and dual-channel heater controller (models SF-28 and TC-344B, Warner Instruments). After 10 min of initial perfusion to remove extracellular dye, [Ca$^{2+}$]$_i$ was measured using a xenon arc lamp, interference filters, electronic shutters, x20 fluorescence objective, and a cooled charge-coupled device imaging camera, and determined at 30- to 60-s intervals from the ratio of Fura-2 fluorescence emitted at 510 nm with excitation at 340 nm to that with excitation at 380 nm (F$_{380}$/F$_{340}$). Data were collected online with InCyte software (Intra-cellular Imaging, Cincinnati, OH). Background images were acquired by unfocusing the imaging field. The background values were subtracted from the mean fluorescence intensities at each wavelength before calculation of the ratio values. [Ca$^{2+}$]$_i$ was estimated from standard curves established with calibration solutions containing 0–1,350 nM Ca$^{2+}$ (Invitrogen) and is presented as an average from 20–30 cells.

Measurement of SOCE. PASMCs were perfused for at least 10 min with Ca$^{2+}$-free KRBS containing 1 mM EGTA to chelate residual Ca$^{2+}$, 5 μM nifedipine (Sigma-Aldrich, St. Louis, MO) to prevent calcium entry through L-type VDCC and 10 μM cyclopiazonic acid (CPA, Sigma-Aldrich) to deplete SR Ca$^{2+}$ stores. SOCE was assessed in two ways, as described previously (22, 41). First, [Ca$^{2+}$]$_i$ was measured before and after restoration of extracellular [Ca$^{2+}$]$_i$ to 2.5 mM. SOCE was evaluated from the peak increase in [Ca$^{2+}$]$_i$, caused by restoration of extracellular Ca$^{2+}$ in the continued presence of nifedipine and CPA. Second, we monitored Fura-2 fluorescence excited at 360 nm at 30-s intervals before and after addition of MnCl$_2$ (200 μM) to the cell perfusate; SOCE was evaluated from the rate at which Fura-2 fluorescence was quenched by Mn$^{2+}$, which entered the cell as a Ca$^{2+}$ surrogate and reduced Fura-2 fluorescence upon binding to the dye. Fluorescence excited at 360 nm was the same for Ca$^{2+}$-bound and Ca$^{2+}$-free Fura-2; therefore, changes in fluorescence were caused by Mn$^{2+}$ alone.

DNA extraction and real-time quantitative PCR. Total RNA in PASMCs was extracted using RNeasy kit (Qiagen, Valencia, CA). DNA contamination in RNA preparations was removed by on-column DNase digestion using RNeasy column and RNase-free DNaseI (Qiagen). Transcript mRNA was designed and synthesized by Dharmacon (Lafayette, CO). It is a siGENOME SMARTpool containing four individual duplexes targeting to different areas of BMP4 mRNA (accession no. NM_012827). Nontargeting siRNA Pool (Dharmacon) served as a negative control.

The program of real-time quantitative PCR consisted of a hot start at 95°C for 15 min, 45 cycles with each containing 94°C for 15 s, 57.5°C for 20 s, and melting curves performed at 95°C for 15 min, 55°C for 1 min, and an increment of 0.5°C for 80 repeats. Specificity of the PCR products was verifiably by melting curves, agarose gel electrophoresis, and DNA sequencing. Detection threshold cycle (Ct) values were generated by iCyclerIQ software. PCR efficiency of each pair of primers was obtained using the Pfaffl method (32). TRPC or BMP mRNA copies were normalized to cyclophilin B and are expressed as percent change from control values.

Western blot analysis. PASMCs following various treatments were scraped and lysed in ice-cold Laemmli sample buffer containing 62.5 mM Tris·HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% protease inhibitor cocktail, 1 mM EDTA, and 200 μM 4-((N-morpholino)loxymethyl)benzenesulfonyl fluoride hydrochloride. Cell lysate proteins were quantified using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). BMP2 and BMP4 proteins in cell-conditioned media were pulled down using heparin-conjugated Sepharose bead and were suspended in sample buffer. Cell lystate or conditioned media proteins were denatured by adding dithiothreitol to 150 mM and heating at 95°C for 3 min and resolved by 10% SDS-PAGE. Separated proteins were transferred onto polyvinylidene difluoride membranes (pore size 0.45 μM, Bio-Rad). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween-20.

Measurement of SOCE. PASMCs were perfused for at least 10 min with Ca$^{2+}$-free KRBS containing 1 mM EGTA to chelate residual Ca$^{2+}$, 5 μM nifedipine (Sigma-Aldrich, St. Louis, MO) to prevent calcium entry through L-type VDCC and 10 μM cyclopiazonic acid (CPA, Sigma-Aldrich) to deplete SR Ca$^{2+}$ stores. SOCE was assessed in two ways, as described previously (22, 41). First, [Ca$^{2+}$]$_i$ was measured before and after restoration of extracellular [Ca$^{2+}$]$_i$ to 2.5 mM. SOCE was evaluated from the peak increase in [Ca$^{2+}$]$_i$, caused by restoration of extracellular Ca$^{2+}$ in the continued presence of nifedipine and CPA. Second, we monitored Fura-2 fluorescence excited at 360 nm at 30-s intervals before and after addition of MnCl$_2$ (200 μM) to the cell perfusate; SOCE was evaluated from the rate at which Fura-2 fluorescence was quenched by Mn$^{2+}$, which entered the cell as a Ca$^{2+}$ surrogate and reduced Fura-2 fluorescence upon binding to the dye. Fluorescence excited at 360 nm was the same for Ca$^{2+}$-bound and Ca$^{2+}$-free Fura-2; therefore, changes in fluorescence were caused by Mn$^{2+}$ alone.

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Tween 20 and blotted with affinity-purified rabbit polyclonal antibodies specific for TRPC proteins, goat polyclonal antibody to BMP2, or mouse monoclonal antibodies to BMP4 or \( \text{\textsuperscript{1}}\)-actin. Bound antibodies were probed with horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and detected using an enhanced chemiluminescence system (ECL, GE healthcare, Piscataway, NJ).

**Drugs and materials.** Unless otherwise specified, all reagents were obtained from Sigma-Aldrich. TRPC antibodies other than TRPC1 were obtained from Alomone Laboratories (Jerusalem, Israel). BMP2 and BMP4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). rhBMP4 stock solutions at 50 \( \mu \text{g/ml} \) were made in 4 mM HCl containing 0.1% BSA. Stock solutions (30 mM) of CPA and nifedipine were made in dimethyl sulfoxide (DMSO). Fura-2 AM

### Table 1. Real-time qPCR primers for rat TRPC, BMP, and cyclophilin B

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<th>Gene</th>
<th>Accession No.</th>
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<th>Location in Sequence</th>
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<td>118</td>
<td>170–287</td>
</tr>
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qPCR, quantitative PCR; BMP, bone morphogenetic protein; TRPC, canonical transient receptor potential.

**Fig. 1.** 

A: expression of canonical transient receptor potential (TRPC) mRNA relative to cyclophilin B in pulmonary arterial smooth muscle cells (PASMCs) treated with bone morphogenetic protein 4 (BMP4) at 5 ng/ml, 50 ng/ml, 250 ng/ml, or vehicle (control) for 60 h as determined by real-time quantitative PCR (\( n = 4 \) in each group). B: expression of TRPC mRNA relative to cyclophilin B in PASMCs treated with 50 ng/ml BMP4 or vehicle control for 24 h, 48 h, or 60 h as determined by real-time quantitative PCR (\( n = 4 \) in each group). Data are presented as percent change from control. \( *P < 0.05 \) vs. vehicle control cells.
was prepared on the day of the experiment as a 2.5 mM stock solution in DMSO containing 20% pluronic F-127 (Invitrogen).

Statistical analysis. Data are expressed as means ± SE, and $n$ is the number of experiments performed, which equals the number of animals providing cells. Statistical analyses were performed using Student’s $t$-test. Differences were considered significant when $P < 0.05$.

RESULTS

Treatment with BMP4 increased TRPC expression in PASMCs. Treatments of BMP4 at 5 ng/ml, 50 ng/ml, or 250 ng/ml for 60 h dose dependently increased the mRNA expression of TRPC1, TRPC4, and TRPC6 in PASMCs (Fig. 1A). The respective inductions of these three transcripts by 50 ng/ml BMP4 treatments were not significantly different. Therefore, the optimal dosage of BMP4 for maximal induction was considered to be 50 ng/ml, which caused increases of mRNA expression of TRPC1, TRPC4, and TRPC6 by 166.2%, 230.3%, and 180.9%, respectively (Fig. 1A). Under the optimal treatment condition (50 ng/ml, 60 h), BMP4 also increased the protein expression of TRPC1, TRPC4, and TRPC6 in PASMCs, as confirmed by Western blotting (Fig. 2).

Treatment with BMP4 increased SOCE in PASMCs. SOCE was assessed in two ways, Ca$^{2+}$ restoration and Mn$^{2+}$ quenching. In the Ca$^{2+}$ restoration assessment, CPA given in the absence of extracellular Ca$^{2+}$ and presence of nifedipine caused an initial transient increase in [Ca$^{2+}$], indicating Ca$^{2+}$ release which was not different between control PASMCs and cells treated with BMP4 (Fig. 3A). Subsequent restoration of extracellular Ca$^{2+}$ induced a second increase in [Ca$^{2+}$], which quickly achieved a peak $\Delta$[Ca$^{2+}$], of 373.8 ± 53.7 nM ($n = 4$) in PASMCs with BMP4 treatment; this peak change in [Ca$^{2+}$], was greater compared with that measured in vehicle control cells ($\Delta$[Ca$^{2+}$], 210.4 ± 36.7 nM, $n = 4$) ($P < 0.05$; Fig. 3A).

Mn$^{2+}$ quenching, which is measured as the rate at which Mn$^{2+}$ quenched Fura-2 fluorescence, is thought to be a more specific index of Ca$^{2+}$ influx. SOCE evaluated by Mn$^{2+}$ quenching and expressed as the percent decrease in fluorescence at time 10 min from time 0 of perfusion was greater in BMP4-treated PASMCs (55.0 ± 1.7%, $n = 3$) compared with that in vehicle control cells (30.3 ± 1.2%, $n = 3$; $P < 0.001$; Fig. 3B).

Treatment with BMP4 increased basal [Ca$^{2+}$] in PASMCs. Basal [Ca$^{2+}$], was increased from 112.4 ± 7.4 nM ($n = 16$) in control cells exposed to vehicle (4 mM HCl with 0.1% BSA) to 255.6 ± 5.2 nM ($n = 16$) in PASMCs treated with 50 ng/ml BMP4 for 60 h ($P < 0.001$; Fig. 4A). Removal of extracellular Ca$^{2+}$ by perfusion with Ca$^{2+}$-free KRBS containing 1 mM EGTA for 10 min did not affect basal [Ca$^{2+}$], in control cells ($n = 4$); however, it caused a 75.6 ± 10.5 nM reduction of
[Ca\textsuperscript{2+}] in cells treated with BMP4 (n = 4; P < 0.05 vs. control) (Fig. 4B). Moreover, perfusion with KRBS containing SOCC antagonist SKF-96365 (50 μM) or NiCl\textsubscript{2} (500 μM) for 10 min in PASMCs treated with BMP4 decreased basal [Ca\textsuperscript{2+}], about 87.3 ± 31.7 nM (n = 4; P < 0.05 vs. control) or 84.5 ± 16.5 nM (n = 4; P < 0.05 vs. control), respectively; however, neither SKF-96365 nor NiCl\textsubscript{2} altered basal [Ca\textsuperscript{2+}], in control cells (Fig. 4, C and D).

Knockdown of BMP4 reduced TRPC expression, SOCE, and basal [Ca\textsuperscript{2+}], in serum-stimulated PASMCs. In this study, we found that BMP4 is endogenously produced by serum-stimulated proliferating PASMCs; therefore it could act on cells via an autocrine mechanism (Fig. 5, A and B). To verify the above effect of BMP4 on Ca\textsuperscript{2+} signaling in PASMCs, we used siRNA targeted to BMP4 (BMP4 siRNA) to knock down BMP4 expression in the culture. The specificity of BMP4

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Fig. 4. A: changes in basal [Ca\textsuperscript{2+}], in rat distal PASMCs treated with 50 ng/ml BMP4 (n = 16 from 365 cells) for 60 h. Bar values are means ± SE. *P < 0.001 vs. vehicle control cells (n = 16 from 358 cells). B: removal of extracellular Ca\textsuperscript{2+} reduced basal [Ca\textsuperscript{2+}], in rat distal PASMCs treated with BMP4 (50 ng/ml, 60 h) (n = 4 in 115 cells), but not in control cells treated with vehicle (n = 4 in 136 cells). C: store-operated calcium channel (SOCC) antagonist SKF-96365 and NiCl\textsubscript{2}, attenuated basal [Ca\textsuperscript{2+}], in rat distal PASMCs treated with BMP4 (50 ng/ml, 60 h) (n = 4 in 109 cells), but not in control cells treated with vehicle (n = 4 in 121 cells). D: SOCC antagonist attenuated basal [Ca\textsuperscript{2+}], in PASMCs treated with BMP4 (50 ng/ml, 60 h) (n = 4 in 112 cells), but not in control cells treated with vehicle (n = 4 in 119 cells). Values are means ± SE. *Significantly different from vehicle control: P < 0.05.
siRNA was verified by determining the expression of BMP2, which shares high sequence homology and hence belongs to the same subgroup with BMP4 in the BMP family. As seen in Fig. 5, A and B, exposure of PASMCs to BMP4 siRNA resulted in 94.3% reduction of BMP4 mRNA and in significant decreases of BMP4 protein in conditioned media compared with that in nontargeting siRNA (NT siRNA)-treated cells. Treatment with BMP4 siRNA did not alter BMP2 expression in PASMCs, as demonstrated by real-time PCR for its mRNA (Fig. 5A), and Western blotting for its protein level in the cell conditioned media (Fig. 5B), suggesting a specific knockdown of BMP4 in PASMCs. Next, we examined the effect of decrement in BMP4 on TRPC expression, and the subsequent functional influences on SOCE and basal \([\text{Ca}^{2+}]_i\) in rat distal PASMCs. As indicated in Fig. 5, C and D, knockdown of BMP4 by BMP4 siRNA caused significant decreases of mRNA (Fig. 5C) and protein (Fig. 5D) expression of TRPC1, TRPC4, and TRPC6 compared with that in cells treated with NT siRNA. Consequently, SOCE was decreased by 52.3% (P < 0.001, Fig. 6A) and 46.4% (P < 0.05; Fig. 6B) as measured by \([\text{Ca}^{2+}]_i\), responses to extracellular \([\text{Ca}^{2+}]_i\) restoration and Mn\(^{2+}\) quenching, respectively, in PASMCs treated with BMP4 siRNA. In addition, basal \([\text{Ca}^{2+}]_i\), was reduced from 166.0 ± 7.5 nM (n = 4) in cells treated with NT siRNA to 106.9 ± 8.1 nM (n = 4) in cells treated with BMP4 siRNA (P < 0.01; Fig. 7).

**DISCUSSION**

This study presents important evidence indicating that BMP4 acts as a regulator of \([\text{Ca}^{2+}]_i\) signaling in PASMCs.

First of all, we found that treatment with BMP4 increased TRPC1, TRPC4, and TRPC6 expression in growth-arrested PASMCs. The function of TRPC1, TRPC4, and TRPC6 proteins has been mainly interpreted as components of SOCC, mediating SOCE, thus regulating diverse cellular activity, such as proliferation, migration, and contraction in response to various stimuli. Upregulated TRPC1, TRPC4, and TRPC6
expression was found to correlate with increased SOCE in proliferate PASMCs (12, 38). Overexpression of human TRPC1 enhanced contractile responses to CPA in rat pulmonary arterial rings (18). PASMCs treated with PDGF or ATP exhibited an increase in TRPC6 and TRPC4 expression, correlating with enhanced SOCE (50, 53). In contrast, reduction of TRPC1, TRPC4, or TRPC6 with antisense oligonucleotide, siRNA, bosentan, or sildenafil resulted in attenuated amplitude of SOCE induced by mitogen or hypoxia in human or rat PASMCs (17, 23, 38, 50, 53).

If TRPC1, TRPC4, and TRPC6 are indeed components of SOCC in PASMCs, the increased expression of these Ca2+ channel proteins could increase the number of SOCC and lead to enhanced SOCE in cells treated with BMP4. Therefore, we next tested whether treatment with BMP4 indeed influenced SOCE in PASMCs. Measurement of SOCE via [Ca2+]i decrease in fluorescence from distal PASMCs treated with BMP4 siRNA (n = 3 experiments in 86 cells) or NT siRNA (n = 3 experiments in 87 cells). Bar graph indicates that maximum increase in [Ca2+]i, after restoration of extracellular [Ca2+]i, was reduced in BMP4 siRNA-treated PASMCs compared with that in NT siRNA-treated cells (*P < 0.001). A: time course of quenching of Fura-2 fluorescence at 360 nm by 200 μM Mn2+ after perfusion with Ca2+-free KRBS (0 Ca2+) containing CPA (10 μM), nifedipine (Nifed; 5 μM), and EGTA (1 mM) in rat distal PASMCs treated with BMP4 siRNA (n = 3 experiments in 86 cells) or NT siRNA (n = 3 experiments in 87 cells). Bar graph indicates that maximum increase in [Ca2+]i, after restoration of extracellular [Ca2+]i, was reduced in BMP4 siRNA-treated PASMCs compared with that in NT siRNA-treated cells (*P < 0.05). Bar values are means ± SE.

Also used Mn2+ quenching of fluorescence excited at 360 nm (F360) as a more direct evaluation of Ca2+ entry. Following store depletion with CPA, Mn2+ caused significantly greater quenching of F360 in PASMCs from BMP4 treatment compared with that in cells from vehicle control treatment. The increased amplitude of [Ca2+]i, following restoration of extracellular Ca2+ and faster rate of Mn2+ quenching of F360 in cells from BMP4 treatment indicate enhanced Ca2+ influx through SOCC. One possible explanation for this increased SOCE is that it results from an increased number of SOCC due to increased expression of TRPC. Another possibility is a greater degree of store depletion; however, the increase of [Ca2+]i in response to CPA was not significantly different in vehicle and BMP4-treated PASMCs, suggesting equal depletion of stored Ca2+. Hence, differences in store depletion do not account for enhanced SOCE caused by BMP4. Our data suggest that the enhanced SOCE in cells treated with BMP4 could indeed be due to greater number of SOCC. Finally, we found that BMP4 treatment enhanced basal [Ca2+]i, in PASMCs. This increase in basal [Ca2+]i, was abolished by removal of extracellular Ca2+ or perfusing cells with SOCC inhibitor SKF-96365 (50 μmol/l) or NiCl2 (500 μmol/l) at concentrations that inhibited SOCE by >80% (41), demonstrating the requirement of Ca2+ influx through SOCC.

Contrary to the upregulatory effect of BMP4, knockdown of endogenous BMP4 expression in these cells by specific siRNA resulted in reduced expression of TRPC1, TRPC4, and TRPC6 and consequently reduced SOCE and basal [Ca2+]i, in proliferating rat PASMCs stimulated with serum, confirming the regulatory role of BMP4 in expression of TRPC and Ca2+ signaling in PASMCs.

Substantial evidence indicates that the rise of [Ca2+]i, is an important trigger for pulmonary vascular medial hypertrophy by stimulating PASMC proliferation and migration (2, 33). Mitogenic stimulation of PASMCs leads to elevated [Ca2+]i, by triggering Ca2+ release from the SR and Ca2+ influx through SOCC (2, 4, 7, 14, 15, 30, 34, 54). Removal of extracellular Ca2+ and chelation of intracellular Ca2+ markedly inhibited PASMC proliferation induced by serum or growth factors, e.g., PDGF (50, 38). While other BMPs, such as BMP2 and BMP7, seem to be mainly apoptotic or inhibitory to PASMC proliferation, BMP4 was found to stimulate proliferation and migration of mouse PASMCs, and human cells from peripheral...
Heterozygous BMP4-null mice were absent of hypoxic increases of BMP4 expression and protected from hypoxia-induced vascular smooth muscle cell proliferation and vascular remodeling during development of CPH (10). Our findings of the enhancement of expression of TRPC1, TRPC4, and TRPC6 by BMP4, and, consequently, basal [Ca\(^{2+}\)]\(_i\) elevation in PASMCs, suggest that the BMP4 regulation of PASMC proliferation and pulmonary vascular remodeling is likely mediated through increases in basal [Ca\(^{2+}\)]. In contrast to the enhancing effects of BMP4, there were also other observations indicating that BMP4 is anti-proliferative or apoptotic to PASMCs (19, 48). The contradictory effects of BMP4 in PASMCs from different studies likely reflect that the specific effect of BMP4 varies depending on the functional receptors it primarily binds to when tested in cells from different anatomic location, or the system the culture takes place.

In common with other BMPs, BMP4 transduces its signals through type I and type II receptors, leading to the activation of Smad and MAPK pathways (47, 48). Normally, BMP receptor 2 (BMPR2) is the major type II receptor that responds to BMP4. Studies have suggested that ablation of BMPR2 may facilitate the binding of BMP4 to other type II receptors such as ActRII through the utilization of coreceptor repulsive guidance molecule RGMa and lead to attenuated activation of Smad1/5, without disrupting the activation of p38MAPK and ERK1/2 in PASMCs (35, 46, 47). Activation of Smad signaling has been confirmed to be associated with growth inhibition and apoptosis, while activation of p38MAPK/ERK leads to enhanced proliferation of PASMCs (19, 47, 48). Further study is needed to validate whether our data obtained in cell cultures are relevant to reality in vivo with respect to the development of CPH, and to further elucidate the signaling pathways involved in the upregulation of TRPC by BMP4, particularly in cells anchored with loss-of-function BMPR2 mutant.

In summary, this is the first report, to our knowledge, to show that BMP4 regulates TRPC expression, SOCE, and basal [Ca\(^{2+}\)]\(_i\) elevation in PASMCs, and to further elucidate the signaling pathways responsible for the antiproliferative or apoptotic effects of BMP4 in PASMCs. Our findings of the enhancement of expression of TRPC1, TRPC4, and TRPC6 by BMP4, and, consequently, basal [Ca\(^{2+}\)]\(_i\) elevation in PASMCs, suggest that the BMP4-mediated activation of receptors coupled by the Gq class of G protein.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

References

BMP4 increases TRPC expression in pulmonary arterial smooth muscle


