

Parathyroid hormone promotes osteoblastic differentiation of endothelial cells via the extracellular signal-regulated protein kinase 1/2 and nuclear factor- κ B signaling pathways

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Abstract. Vascular calcification (VC) occurs in patients with chronic kidney disease (CKD) and contributes to cardiovascular dysfunction and mortality. Parathyroid hormone (PTH) is a crucial regulator of VC. High PTH serum levels constitute as a major risk factor for patients with CKD. However, the effect and mechanism of PTH on osteoblastic differentiation in endothelial cells have not been fully elucidated. In the present study, the role of PTH in VC was investigated using an *in vitro* calcification model. Endothelial cells were stimulated with PTH in the femto- to picomolar range. As determined by western blot analysis and ELISA, osteoblastic differentiation, as indicated by the BMP2 marker, occurred with maximum effect at 1×10^{-10} mmol/l PTH. The results indicate that PTH promotes osteoblastic differentiation of endothelial cells, as demonstrated by the increased expression of bone morphogenetic protein (BMP) 2 and BMP4. In addition, western blot analysis revealed that PTH activated the extracellular signal-regulated protein kinase (Erk)1/2 and nuclear factor (NF)- κ B signaling pathways. However, reverse transcription-quantitative polymerase chain reaction demonstrated that inhibitors specific to Erk1/2 and NF- κ B eradicated the effect of PTH treatment on BMP2, BMP4, ALP and RUNX2 expression. These results demonstrate that PTH promotes the osteoblastic differentiation of endothelial cells via the Erk1/2 and NF- κ B signaling pathways, which suggests a potential role of PTH in the promotion of VC. These findings provide an insight into the association between PTH and cardiovascular disease.

Introduction

Vascular calcification (VC) is associated with cardiovascular-related mortality in patients with chronic kidney disease (CKD) (1). VC is considered to be a passive and degenerative process. However, previous findings have indicated that VC may be a pathobiological process with respect to embryonic bone formation. It has been demonstrated in animal models that VC may be induced by stimulating various genes, including bone morphogenetic protein (BMP)2, BMP4 (2,3), runt-related transcription factor (RUNX)2 and alkaline phosphatase (ALP) (4). Previous studies have revealed that BMP2 and BMP4, members of the transforming growth factor- β cytokine superfamily, participate in vascular calcification (5). Vascular endothelial and smooth muscle are key sources of BMPs, which when detected in calcified blood vessels may be associated with enhanced vascular calcification (6).

Parathyroid hormone (PTH) is a critical bridge between bone metabolism and cardiovascular disease (7). PTH regulates plasma calcium concentration and maintains skeletal integrity (8). PTH receptors are present throughout the cardiovascular system, including in smooth muscle cells, endothelial cells and cardiomyocytes (9-11). PTH participates in the development of VC, which is elevated in patients with chronic renal failure (12). PTH may therefore serve a role in the pathophysiology of cardiovascular disease (13). A series of previous studies (14,15) have suggested that PTH may directly induce endothelial dysfunction and contribute to VC. However, the underlying mechanisms of this remain unclear.

In the present study, it was hypothesized that PTH may be associated with the regulation of osteoblastic differentiation in endothelial cells. To determine its effects and develop a model for the mechanism of action, PTH was used to induce calcification of endothelial cells in culture as previously described (16). The effect of PTH on the osteoblastic differentiation of endothelial cells and the associated cell signaling pathways, Erk1/2 and NF- κ B, was also examined. Elucidating the role of PTH may provide critical information concerning bone disease as well as novel preventive and therapeutic opportunities for patients with cardiovascular disease.

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Materials and methods

Ethical statement. The present study was approved by the Animal Ethics Committee of Anhui Medical University (Wuxi, China).

Cell culture and treatment. Primary human umbilical vein endothelial cells (HUVECs) were purchased (cat. no. JL10025; Shanghai Jianglin Biotechnology Company, Shanghai, China) and cultured according to the manufacturer's recommended protocol. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). DMEM was replaced daily. When the cultures reached 80% confluence, adherent cells were detached using 0.05% trypsin and 0.03% EDTA in PBS for 2 min at 37°C. The collected cells (1×10⁵ cells/well) were cultured in DMEM with 5% CO₂. The cells were washed once with PBS for 2 min, resuspended in DMEM and aliquoted into 12-well plates at 37°C at a density of 1×10⁶ cells/well. Confluent cultures of HUVECs were passaged 3–4 times and subsequently incubated with various concentrations (10⁻¹¹, 10⁻¹⁰, 10⁻⁹ and 10⁻⁸ mmol/l) of a PTH peptide fragment (teriparatide; residues 1–34; R&D Systems, Inc., Minneapolis, MN, USA) at 37°C for 72 h. Western blot analysis and ELISA analysis were subsequently performed to detect BMP2 and BMP4. HUVECs without PTH treatment were used as a control.

Additionally, HUEVC's were incubated with 10⁻¹⁰ mmol/l PTH for increasing time periods (5–60 min) to investigate the effect of PTH on the Erk1/2 and NF-κB signaling pathways. The cells were seeded at 1×10⁵ cells/well in 24-well plates and cultured at 37°C.

HUVECs were also incubated with Erk1/2 inhibitor, U0126 and NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) (both Tocris Bioscience, Bristol, UK). There were three treatment groups and the HUEVCs were incubated with U0126 (1 μM), PDTC (10 μM) or U0126 (1 μM)+PDTC (10 μM) for 2 h prior to treatment with 10⁻¹⁰ mmol/l PTH for 72 h. The cells were seeded at 1×10⁵ cells/well and the inhibitors were added to the cells at 37°C.

Western blot analysis. Western blot analysis was used to detect BMP2, BMP4, phosphorylated and total Erk1/2, and phosphorylated and total NF-κB. β-actin was used as a loading control. Following PTH stimulation for 72 h, HUVECs were lysed with ice-cold radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.). Lysates were subjected to centrifugation at 12,000 × g for 10 min at 4°C. Protein concentration in the supernatant was measured using a BCA kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Total protein (20 μg/lane), which was extracted from the HUVECs was separated by 10–12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA), which was blocked with 5% bovine serum albumin (cat. no. ST023; Beyotime Institute of Biotechnology, Haimen, China) in PBS for 1 h at room temperature (25°C). Blots were exposed to primary antibodies directed against BMP2 (1:1,000; cat. no. ab183729), BMP4 (1:2,000; cat. no. ab124715) (both Abcam, Cambridge, UK), Erk1/2 (1:1,000; cat. no. 4695),

NF-κB (1:1,000; cat. no. 8242), phospho-NF-κB (1:1,000; cat. no. 3033), phospho-Erk1/2 (1:1,000; cat. no. 4370), and β-actin (1:1,000, cat. no. 4970) (both CST Biological Reagents Co., Ltd., Shanghai, China) overnight at 4°C. Following washing three times with tris-buffered saline with Tween-20 for 10 min each time, the membranes were incubated with anti-rabbit immunoglobulin G, horseradish peroxidase-linked antibodies (1:3,000; cat. no. 7074; CST Biological Reagents Co., Ltd.) for 1 h at room temperature. Thereafter, signals were detected using a chemiluminescence detection kit (Merck KGaA, Darmstadt, Germany). The relative protein expression levels were measured using the accompanying computerized image analysis program (ImageQuant LAS 4000 version 1.2; GE Healthcare Life Sciences, Little Chalfont, UK).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from HUVECs was extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA using a PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocols. RT-qPCR was performed using SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol, on a CFX96-Real-Time system. Sample preparations whose 260/280 nm absorbance ratio fell between 1.8 and 2.0 were selected as templates. Genecopoeia (Rockville, MD, USA) designed and synthesized the primers. The RT-qPCR primers were as follows: BMP2 forward, 5'-TCAAGCCAAACACACAAACAGC-3' and reverse, 5'-ACGTCTGAACAATGGCATGA-3'; RUNX2 forward, 5'-CCGGTCTCCTTCCAGGAT-3' and reverse, 5'-GGGAAGTGTCTGTGTGGCTTC-3'; ALP forward, 5'-TGTTCTCTGGGAGATGGTATG-3' and reverse, 5'-CCTGAGCGTTGGTGTGTGTA-3'; BMP4 forward 5'-CACCTCAACTCAACCAACCA-3' and reverse, 5'-CAACACCACCTTGTCATACTCA-3'; GAPDH forward, 5'-GGCTGCCCA GAACATCAT-3' and reverse, 5'-CGGACACATTGGGGG TAG-3'. The PCR conditions included an initial denaturation for 5 sec at 95°C, followed by 40 cycles of denaturation for 10 sec at 95°C, annealing for 20 sec at 60°C and extension for 15 sec at 72°C. All experiments were repeated in triplicate. The relative expression levels of mRNA were calculated with the 2^{-ΔΔCq} method (17) and expression was normalized to that of GAPDH.

ELISA analysis. Levels of BMP2 [cat. no. EK11082-96T; Hangzhou MultiSciences (Lianke) Biotech, Co., Ltd., Hangzhou, China] and BMP4 (cat. no. DBP400; R&D Systems, Inc., Minneapolis, MN, USA) in supernatants were analyzed using ELISA kits according to the manufacturer's protocol. Absorbance was read at 450 nm on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results were determined within the linear range of the standard curve.

Statistical analysis. Data are presented as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Each treatment group was compared with the control group using the Dunnett's test or one-way analysis of variance with the post hoc Bonferroni or Dunn test to analyze differences between experimental groups. P<0.05

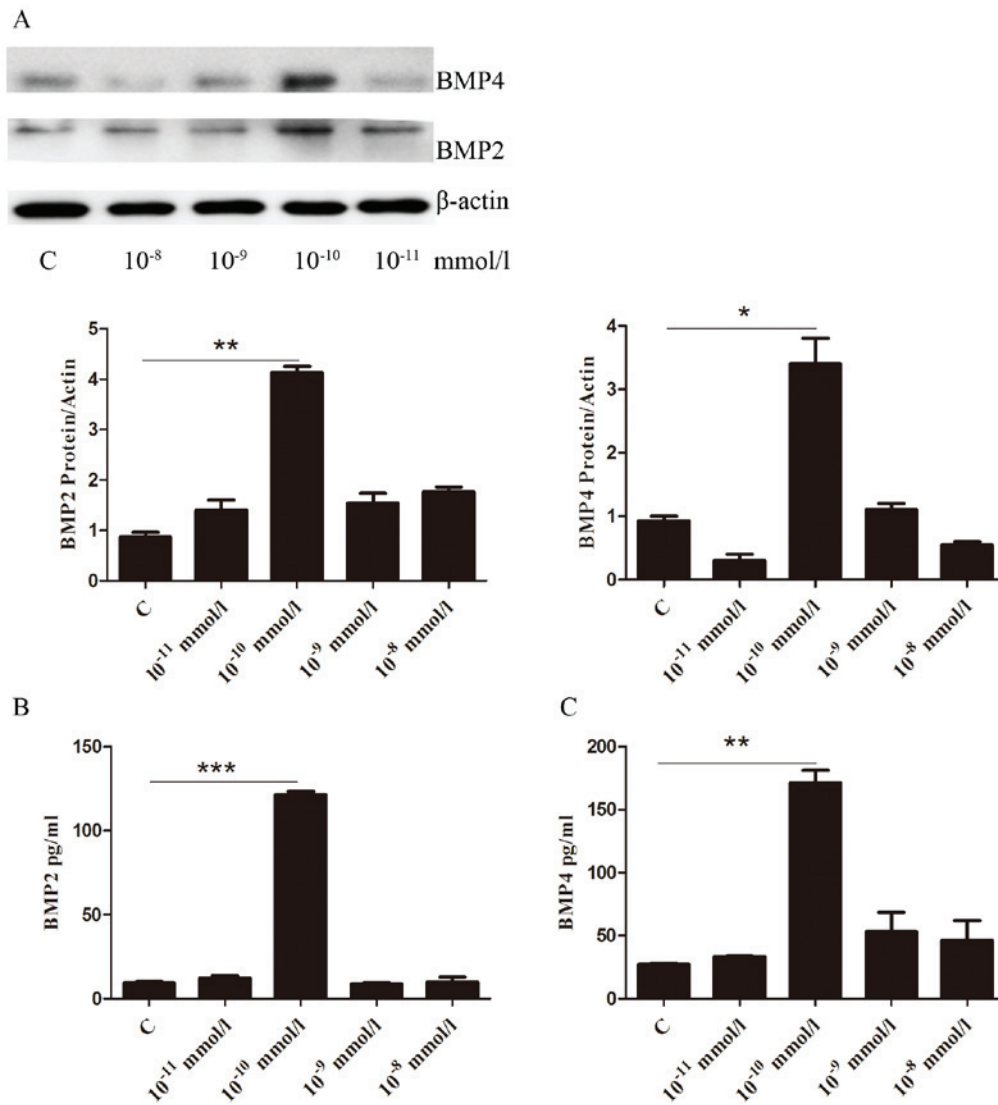


Figure 1. PTH upregulates the production of BMP2 and BMP4 in HUVECs. HUVECs were treated with 10^{-8} to 10^{-11} mmol/l of PTH for 72 h. (A) Representative immunoblots of three separate experiments and normalized densitometry data indicated that 10^{-10} mmol/l of PTH resulted in the greatest expression levels compared with the control. However, concentrations greater than 10^{-10} mmol/l PTH had no additional effect compared with the control. β -actin was used as an internal control. (B) Determination of BMP2 concentration using ELISA from HUVECs treated with PTH. (C) Determination of BMP4 concentration by ELISA from HUVECs treated with PTH. BMP2 and BMP4 levels increased significantly compared with the control after 10^{-10} mmol/l PTH stimulation. $n=3$. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. PTH, parathyroid hormone; BMP, bone morphogenetic protein; HUVECs, human umbilical vein endothelial cells; C, control cells with no PTH treatment.

was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

PTH promotes osteoblastic differentiation of HUVECs. Recent data has suggested that VC occurs as a result of osteoblastic differentiation of HUVECs, in which BMP2 and BMP4 are significantly upregulated (18). In the present study, the expression levels of BMP2 and BMP4 were used as markers to indicate osteoblastic differentiation of HUVECs as previously described (19). Results indicated that 10^{-10} mmol/l of PTH resulted in the greatest expression of BMP2 ($P<0.01$) and BMP4 ($P<0.05$) compared with the control (Fig. 1). However, concentrations $>10^{-10}$ mmol/l PTH had no significant effect compared with the control (Fig. 1). The results indicate that a maximal effect was achieved when the PTH concentration was 10^{-10} mmol/l.

Furthermore, the increased expression of BMP2 and BMP4 following PTH stimulation suggests that PTH may promote osteoblastic differentiation.

PTH activates the Erk1/2 and NF- κ B signaling pathways. The Erk1/2 and NF- κ B signaling pathways serve a crucial role in controlling cell differentiation (20). Activation of these signaling pathways may serve as a marker for VC in HUVECs. The results demonstrate that treatment with 10^{-10} mmol/l PTH for 5 min promotes the phosphorylation of Erk1/2 compared with no treatment, with peak activation occurring at 45 min of exposure, however the levels of phospho-Erk1/2 were significantly decreased at 60 min compared with 45 min exposure. ($P<0.01$; Fig. 2A). Treatment with PTH also increases the phosphorylation of NF- κ B following 5 min of exposure, with peak activation occurring at 45 min ($P<0.01$), the levels of phospho-NF- κ B decreased at 60 min, however the change was not significant (Fig. 2B).

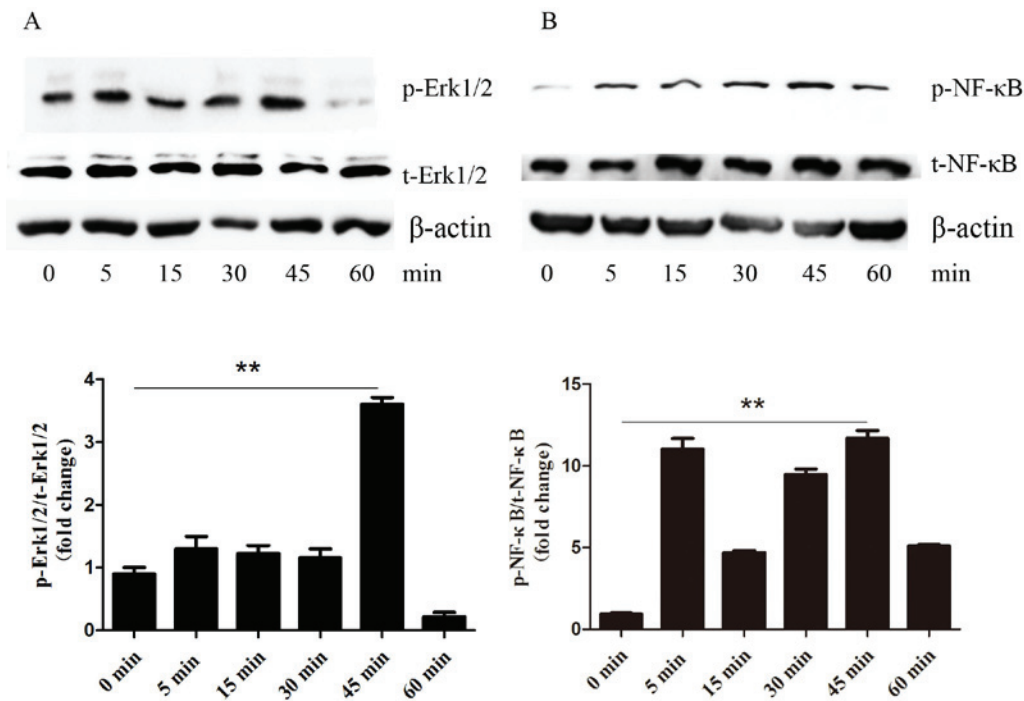


Figure 2. PTH induces the phosphorylation of Erk1/2 and NF- κ B during the osteoblastic differentiation of HUVECs. HUVECs were exposed to 10^{-10} mmol/l PTH for a period of 5-60 min. (A) Representative immunoblots and densitometry data demonstrated that treatment with PTH increased the levels of p-Erk1/2 following 45 min compared with 0 min. (B) Phosphorylation of NF- κ B remained high following 45 min of PTH exposure in HUVECs. $n=3$. ** $P<0.01$ vs. the untreated control (0 min). PTH, parathyroid hormone; Erk, extracellular signal-regulated protein kinase; NF, nuclear factor; HUVECs, human umbilical vein endothelial cells; p, phosphorylated; t, total.

Erk1/2 and NF- κ B signaling mechanisms mediate PTH promotion. The present study demonstrates the protein expression levels of BMP2 and BMP4 following exposure to PTH, PDTC and/or U0126 in HUVECs (Fig. 3A). HUVECs were incubated with U0126 (1 μ M), PDTC (10 μ M) or U0126 (1 μ M)+PDTC (10 μ M) for 2 h prior to treatment with 10^{-10} mmol/l PTH for 72 h. In addition to inhibition of the Erk1/2 and NF- κ B signaling pathways, the PTH-induced overexpression of BMP2 and BMP4 was significantly reduced in endothelial cells following treatment with PDTC and U0126 ($P<0.01$; Fig. 3A and B). Furthermore, PTH-induced BMP2, BMP4, ALP and RUNX2 mRNA overexpression was significantly downregulated as a result of the inhibition of Erk1/2 and NF- κ B signaling pathways ($P<0.01$; Fig. 3C). These findings suggest that PTH may promote calcification via Erk1/2 and NF- κ B signaling in HUVECs.

Discussion

The high prevalence of cardiovascular disease co-occurring with CKD has long been associated with VC (1). A previous study has demonstrated that HUVECs cultured in a high concentration of PTH undergo osteogenic transformation and calcification (21). Furthermore, a previous study indicated that primary and secondary hyperparathyroidism may induce VC (1). PTH is acknowledged for its potent anabolic effect on bone and, furthermore, teriparatide has been used as a therapeutic treatment for patients with severe osteoporosis (22). However, the role of PTH as a risk factor of calcification is not well understood. Low PTH levels have also been reported to be associated with severe arterial calcification (23). Additionally, PTH is known to be a crucial regulator of the cardiovascular system (24).

The results of the present study indicate that PTH may promote the calcification of HUVECs *in vitro*. VC is an actively regulated process that is similar to osteogenesis (25,26). BMP2, a member of the transforming growth factor- β superfamily, regulates osteoblast differentiation and bone formation (27). Overexpression of BMP2 and RUNX2 has been indicated in the calcified vascular smooth muscle cells, which suggests that BMP2 actively regulates the process of VC (6). RUNX2, a key transcription factor belonging to the runt domain gene family, was demonstrated to be overexpressed in the vascular smooth muscle cells (28). These findings suggest that RUNX2 is essential for osteoblast differentiation and may regulate mineralization. Previous results have suggested that HUVECs have acquired phenotypic transitions that are associated with the expression of RUNX2 (21). In the present study, PTH has been demonstrated to promote the calcification of HUVECs by increasing the expression of BMP2, BMP4, RUNX2 and ALP. In a previous study, high levels of PTH induced endothelial to chondrogenic transition in HUVECs (21). Furthermore, these results suggest that PTH may induce the osteoblastic differentiation of HUVECs by mediating the Erk1/2 and NF- κ B signaling pathways.

PTH is an 84-amino acid peptide with only a few interspecies differences, primarily in the amino terminal portion (9,29). The PTH receptor is a typical G protein-coupled receptor with seven transmembrane domains (9). PTH activates either adenylylate cyclase to activate protein kinase A or the phospholipase C protein kinase C signaling pathway (30). PTH overloading contributes to left ventricular hypertrophy, myocardial fibrosis and VC, regardless of arterial pressure (31,32). Furthermore, PTH stimulates the renin-angiotensin-aldosterone system,

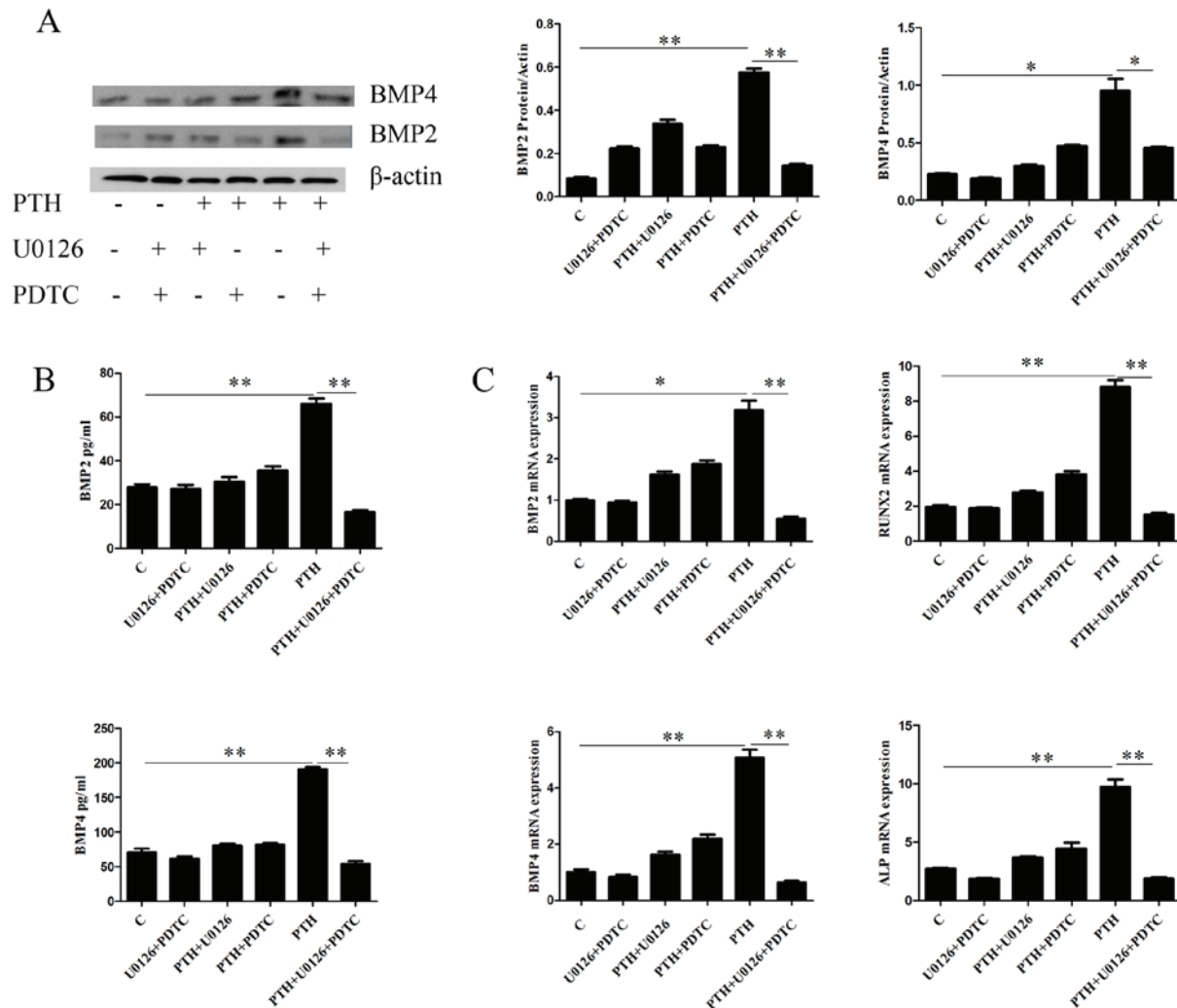


Figure 3. Erk1/2 and NF- κ B are required for the upregulation of BMP2 and BMP4. Endothelial cells were incubated with Erk1/2 inhibitor (U0126, 1 μ M) or NF- κ B inhibitor (PDTC, 10 μ M) or both for 2 h prior to 72 h PTH stimulation. (A) Representative immunoblots and densitometry data indicated that inhibition of either Erk1/2 or NF- κ B or both abrogated the effect of PTH on BMP2 and BMP4 expression levels. (B) BMP2 and BMP4 protein expression levels were determined using ELISA in endothelial cells following the different treatments. (C) Reverse transcription-quantitative polymerase chain reaction analysis was performed to assess BMP2, BMP4, ALP, and RUNX2 mRNA expression levels following the different treatments. n=3. *P<0.05 and **P<0.01. Erk, extracellular signal-regulated protein kinase; NF, nuclear factor; BMP, bone morphogenetic protein; PTH, parathyroid hormone; RUNX, runt-related transcription; ALP, alkaline phosphatase; PDTC, pyrrolidine dithiocarbamate; C, control cells with no PTH treatment.

sympathetic activity and the secretion of cortisol, which may result in sodium and water retention and ultimately hypertension (33). These changes may further induce endothelial dysfunction and promote VC (34). PTH is a crucial regulator of calcium balance in physiological and pathological conditions associated with cardiovascular events, with a major physiological role in bone homeostasis (35,36). Clinically, patients with CKD suffer from high cardiovascular mortality and adverse cardiovascular events, including coronary microvascular dysfunction, subclinical aortic valve calcification, increased aortic stiffness, endothelial dysfunction and hypertension (37,38). Furthermore, PTH reduces VC mediated by the BMP2-Msh Homeobox 2-Wnt signaling pathway (39-41).

In the present study, HUVECs were cultured with different concentrations of PTH. HUVECs were incubated with 10^{-8} to 10^{-11} mmol/l PTH and the results suggested that 10^{-10} mmol/l significantly increased BMP2 and BMP4, expression compared with the control, with the greatest effect observed at 10^{-10} mmol/l PTH. In order to gain further insight into the

mechanism by which PTH promotes VC, signaling events were examined. Erk1/2 and NF- κ B have been acknowledged for their essential roles in cell differentiation (42). A previous study has indicated that Erk1/2 and NF- κ B are associated with osteoblastic differentiation and mineralization of HUVECs (20). The results of the present study demonstrate that PTH activates the Erk1/2 and NF- κ B signaling pathways in HUVECs. Treating HUVECs with Erk1/2 and NF- κ B inhibitors prevented the PTH-induced overexpression of BMP2, BMP4, ALP and RUNX2. Treatment with the Erk1/2 and NF- κ B inhibitors U0126 and PDTC reduced the activation of Erk1/2 and NF- κ B in HUVECs, which suggests that the PTH-induced promotion of BMP2 expression may be mediated by the Erk1/2 and NF- κ B signaling pathways.

The physiological concentration of PTH is much lower than those applied in the present study; as such, the concentration investigated was not of the usual physiological range. It is important to mention that the majority of previous studies have been performed with the N-terminal fragment of PTH,

teriparatide, whereas clinical studies use intact PTH (43). As such, mechanistic studies may not reflect clinical conditions. Furthermore, recent studies have indicated that modifications to PTH alter its biological function (43). The carboxyl terminal PTH fragment, which represents 70-95% of circulating PTH, has a specific effect on calcium homeostasis and bone metabolism, which suggests that carboxyl terminal PTH may increase serum calcium and decrease blood phosphorus (43). In addition, circulating PTH partially oxidizes and loses its PTH receptor-stimulating properties, while remaining detectable by immunoassays (44). Therefore, currently available PTH assay systems may not adequately assess PTH-associated cardiovascular diseases in patients.

In conclusion, the results of the present study demonstrate that PTH serves a crucial role in promoting the osteoblastic differentiation of HUVECs, with Erk1/2 and NF- κ B signaling being associated with this process. Furthermore, these findings suggest that PTH may serve a crucial role in promoting VC.

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