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Protein kinase C βII and δ/θ play critical roles in bone morphogenic protein-4-stimulated osteoblastic differentiation of MC3T3-E1 cells

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ABSTRACT

Bone morphogenic protein-4 (BMP-4), one of TGF-β superfamily, is involved in bone and cartilage development, specifically tooth and bone fracture repair. In the present study, the role of protein kinase C (PKC) was investigated in BMP-4-induced differentiation of osteoblast-like MC3T3-E1 cells. PKC inhibitor UCN-01 significantly attenuated the synthesis of osteocalcin, a marker of mature osteoblast phenotype, in a dose-dependent manner as well as blocked osteroblastic differentiation and mineralization in BMP-4-treated MC3T3-E1 cells. Also, UCN-01 suppressed vascular endothelial growth factor (VEGF) production in BMP-4-treated MC3T3-E1 cells. In addition, UCN-01 remarkably suppressed BMP-4-activated PKC βII and PKC δ/θ of PKC family proteins by Western blotting. Consistently, 2-dimensional electrophoresis (2-DE) immunoblotting revealed that UCN-01 inhibited the BMP-4-induced activation of PKC subfamilies in MC3T3-E1 cells. Taken together, our findings suggest that PKC βII and PKC δ/θ mediate BMP-4-induced osteoblastic differentiation.

1. Introduction

Bone morphogenetic proteins (BMPs) are multifunctional cytokines, which belong to the transforming growth factor-β (TGF-β)/BMP superfamily [1,2]. BMPs were originally discovered by their ability to mediate the formation of ectopic bone [1] and play important roles in the early development of vertebrates [3]. Osteoblasts synthesize BMP-4 of the BMP family, that in turn stimulate the activation of alkaline phosphatase and the expression of osteocalcin [4]. Intracellular BMP signaling generally mediates Smad protein family and so the BMP/Smad pathway leading to osteoblast differentiation has been widely explored [5–7]. In addition to Smad proteins, BMP signaling can stimulate other signaling pathways such as the phosphatidylinositol 3-kinase (PI3K)/p70 S6 kinase, p38 mitogen-activated protein kinase (MAPK) cascades [8], and p44/p42 MAPK in osteoblast [9].

PKC family proteins consist of ten isozymes that are divided into three subfamilies conventional (PKCα, β and γ), novel (PKCδ, ε, η and θ), and atypical (PKCι, ζ and N) PKCs [10,11]. PKCs regulate the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine residues on these proteins. Interestingly, Hay and colleagues reported that BMP-2 promoted osteoblastic apoptosis through Smad-independent and protein kinase C (PKC)-dependent signaling pathway in primary human calvaria osteoblasts and immortalized human neonatal calvaria osteoblasts [12]. Lampasso and colleagues recently reported that there is a specific profile of expression of PKC isoforms in differentiating osteoblasts [13].

In the present study, we demonstrate that PKC plays a role in BMP-4 intracellular signaling in murine osteoblast-like cell line MC3T3-E1 by osteocalcin synthesis assay, Western blotting and 2-DE using PKC inhibitor UCN-01.

2. Materials and methods

2.1. Cell cultures and differentiation induction

Murine osteoblast-like cell line MC3T3-E1 was purchased from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in alpha minimum essential medium (α-MEM) containing 10% fetal bovine serum, 1% penicillin streptomycin. Osteoblastic differentiation was induced by adding 5 mM β-glycerophosphate and 50 μM ascorbic acid-2-phosphate to medium.

2.2. Measurement of osteocalcin content

MC3T3-E1 cells were seeded onto 12-well plates at a density of 1×10^5 cells/ml and treated with 50 ng/ml BMP-4 for 0, 24, 48, 72,

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96 or 120 h. The amount of osteocalcin in cultured supernatant was measured by using osteocalcin enzymeimmunoassay (EIA) kit (Biomedical technologies, Soughton, MA) as described by manufacturer's instructions.

2.3. Cytotoxicity assay

The cytotoxic effects of UCN-01 and BMP-4 were measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MC3T3-E1 cells were seeded onto 96-well plates at a density of 1×10^4 cells/ml and treated with various concentrations of UCN-01 in the absence or presence of BMP-4 for 24 h. Then, MTT stock solution (5 mg/ml) was added onto each well and incubated until formazan was constituted. After removing supernatant, the formazan was dissolved in MTT lysis solution (20% SDS/50% dimethylfermamide) and the optical density (OD) was measured using microplate reader (Molecular Devices Co., Silicon Valley, CA.) at 450 nm. Cell viability was calculated as a percentage of viable cells in UCN-01 and/or BMP-4-treated cells versus untreated control by following equation.

Cell viability (%) = [OD (drug) − OD (blank)]/[OD (control) − OD (blank)] × 100.

2.4. Immunofluorescence staining with PKC

MC3T3-E1 cells were seeded onto poly-L-lysine coated glass coverslips at a density of 1×10^5 cells/ml and incubated with or without 50 ng/ml BMP-4 for 48 h. The cells were fixed in chilled methanol for 15 min and rinsed in PBS. After blocking in 0.5% bovine serum albumin and 3% glycerol in PBS for 1 h at room temperature, the cells were incubated with rabbit monoclonal anti-phospho-PKC (Cell signaling, Beverly, MA) for 4 h at room temperature and immunostained with Alexa 488-conjugated secondary antibody (Molecular Probes Inc., Eugene, OR). The slides were washed three times in PBS, mounted with antifade mounting media and visualized under immunofluorescence microscope, AXIO observer A1 (Carl Zeiss, Heidelberg, Germany).

2.5. Alkaline phosphatase staining

Cells were washed with cold PBS, then fixed 2% paraformaldehyde in PBS for 10 min, and stored in 100 mM cacodylic acid buffer (pH 7.4) at 4 °C. Cells were then incubated at 37 °C with freshly prepared alkaline phosphatase substrate solution (100 mM trismaleate buffer (pH 8.4), 2.8% N,N-dimethylformamide, 1 mg/ml Fast Red TR, and 0.5 mg/ml Naphtol AS-MX phosphate). The reaction was terminated after 30 min by removal of the substrate solution and washing with 100 mM cacodylic acid buffer.

2.6. Calcium measurement

Mineralization was assessed by von Kossa staining with 3% $AgNO_3$ for 5 min. MC3T3-E1 cells were collected by scraping into 10 mM tris-buffered saline (pH 7.2) containing 0.2% Triton X-100. An aliquot was removed to determine protein concentration, and the remants were incubated in 0.5 M HCl at 70 °C for 15 min and detected by spectrophotometry (Massachusetts Institute of Technology, USA).

2.7. Western blot analysis

Cell extracts were prepared by using lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM iodoacetamide, and 2 mM PMSF). The extracts were incubated on ice for 30 min, centrifuged (12,000*g*) at 4 °C for 20 min, and the supernatants were collected. Protein concentrations were determined by Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA), and the lysates were separated by electrophoresis on 10% Tris–glycine gels. The proteins were then transferred to Hybond ECL transfer membrane and analyzed with anti-phospho-PKC and total PKC antibodies (Cell Signaling, Beverly, MA).

2.8. Two-dimensional gel electrophoresis immunoblotting

The differentiated MC3T3-E1 cells were treated with 50 ng/ml BMP-4 for 72 h in the absence or presence of 25 nM UCN-01. Cells were washed twice in PBS and lyzed in sample buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% biolyte 3/10 ampholytes, and trace bromophenol blue). Protein samples were loaded onto immobilized pH gradient (IPG) strips and rehydrated with enough volume of sample buffer for 12 h at 20 °C. Rehydrated IPG strips were isoelectric focused by using Protean IEF Cell System (Bio-Rad, Hercules, CA) with three steps, 250 V, 20 min, linear/4000 V, 2 h, linear/4000 V, 10,000 V h, rapid. After electro focused, IPG strips were equilibrated with equilibration buffer I, II (6 M urea, 2% SDS, 0.375 M Tris–HCl (pH8.8), 20% glycerol, and 2% dithiothreitol) for 10 min, then same protocol for Western blot analysis was used as described previously.

After electrophoresis, proteins were by silver staining based on a mass spectrometry compatible staining, and then subjected to immunoblotting as before.

2.9. VEGF measurement

The differentiated MC3T3-E1 cells were stimulated with 50 ng/ml BMP-4 in α-MEM for 72 h in the presence or absence of 25 nM UCN-01. The level of VEGF in the supernatant was measured using Quantikine M mouse VEGF ELISA kit (R&D systems, Inc., Minneapolis, MN) by ELISA.

2.10. Statistical analysis

All values represent means ± standard error (S.E.). The statistically significant difference was calculated by the student's *t*-test. (Sigma Plot®, San Rafael, CA).

3. Results

3.1. Effect of PKC on osteocalcin synthesis in BMP-4-treated MC3T3-E1 cells

BMP-4 is known to stimulate the synthesis of osteocalcin, a biomarker for the bone formation process [4,14]. To clarify this event, the production of osteocalcin was evaluated in the absence or presence of BMP-4 for 72 h by ELISA. As shown in Fig. 1A and B, BMP-4 increased the level of osteocalcin in time- and dose-dependent manners in osteoblast-like MC3T3-E1 cells.

Recent study reported that BMP-2 activates PKC pathway in human neonatal calvaria cells [12] and PKC is regulated by the concentrations of Ca^{2+}, diacylglycerol (DIG), and phospholipid [15]. In the current study, we investigated whether BMP-4 also influences on PKC activation. MC3T3-E1 cells were treated with or without BMP-4 (50 ng/ml) for 48 h and immunofluorescence assay was performed for PKC expression. As shown in Fig. 1C, BMP-4 remarkably induced the expression of PKC on cell membrane. To examine whether PKC is involved in the BMP-induced osteocalcin synthesis, the cytotoxic effect of 7–hydroxy staurosporine (UCN-01), a specific PKC inhibitor was evaluated by MTT assay and the levels of osteocalcin were measured in MC3T3-E1 cells treated

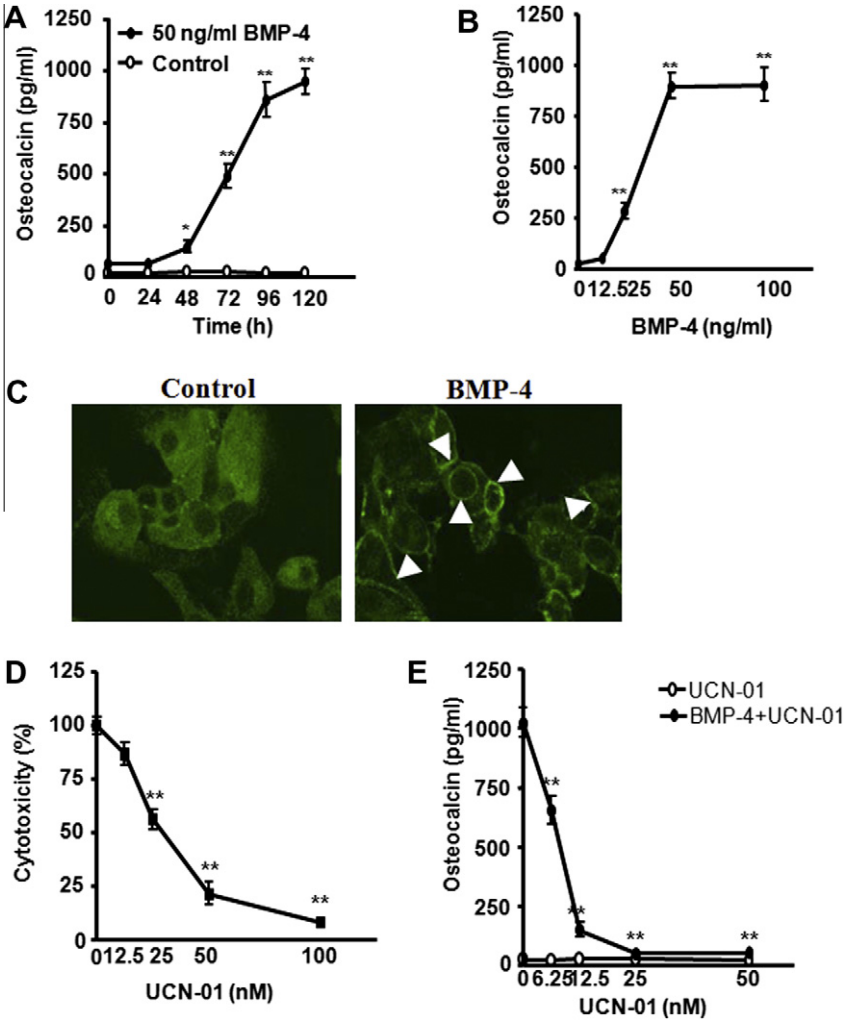

Fig. 1. BMP-4 induces the expression of PKC on cell membrane, and PKC inhibitor UCN-01 exerts cytotoxicity and inhibited osteocalcin synthesis in MC3T3-E1 cells. (A) Osteocalcin synthesis was measured in 50 ng/ml BMP-4-treated MC3T3-E1 cells in a time course (0, 24, 48, 72, 96 and 120 h). (B) Osteocalcin synthesis was measured in 50 ng/ml BMP-4-treated MC3T3-E1 cells in a concentration dependent manner (0, 12.5, 25, 50 and 100 ng/ml) for 72 h by using osteocalcin EIA kit. Data represent means ± S.E., * $p < 0.05$ and **, $p < 0.01$ vs. untreated control. (C) The effect of BMP 4 on PKC expression on cell membrane. Cells were seeded onto glass coverslips at a density of 1×10^5 cells/ml and incubated with or without BMP-4 (50 ng/ml) for 48 h. The cells were fixed and then immunostained with anti-p-PKC primary and Alexa 488-conjugated secondary antibodies. Arrows indicate stained cells. (D) Cytotoxicity of UCN-01 in MC3T3-E1 cells by MTT assay. Cells were seeded on a 96-well plates for at a density of 1×10^4 cells/ml and treated with various concentrations of UCN-01 (0, 12.5, 25, 50 or 100 nM) for 24 h. (E) The effect of UCN-01 on osteocalcin synthesis in BMP-4-treated MC3T3-E1 cells. Cells were pre-treated with various concentrations of UCN-01 (0, 6.25, 12.5, 25 or 50 nM) for 1 h and treated with or without BMP-4 for 72 h. The amounts of osteocalcin were measured using osteocalcin EIA kit. Data represent means ± S.E., ** $p < 0.01$ vs. untreated control.

with BMP-4 and/or UCN-01. UCN-01 significantly reduced the viability of MC3T3-E1 as well as significantly decreased osteocalcin synthesis in a dose-dependent manner as shown in Fig. 1D and E, suggesting that PKC plays an important role in BMP-4-mediated osteocalcin synthesis in MC3T3-E1 cells.

3.2. UCN-01 suppressed osteoblastic differentiation and mineralization in BMP-4 treated MC3T3-E1 cells

To test whether PKC affects on BMP-4-induced osteoblastic differentiation, MC3T3-E1 cells were treated with BMP-4 in the absence or presence of UCN-01 and maintained for 10 days. As shown in Fig. 2A, MC3T3-E1 cells displayed the differentiation morphology in the presence of BMP-4. In contrast, UCN-01 significantly suppressed BMP-4-mediated osteoblastic differentiation.

Alkaline phosphatase acts as a transmembrane receptor involved in osteoprogenitor-osteoblast adhesion, migration and differentiation [16]. Bright red color was more strongly shown in BMP-4 treated group compared to untreated control, suggesting calcium mineralization, while the red color was diluted in UCN-01 and BMP-4-treated group as shown in Fig. 2B.

Similarly, the effect of PKC on BMP-4-mediated osteoblastic mineralization was evaluated by von Kossa staining in MC3T3-E1 cells. As shown in Fig. 2C, UCN-01 significantly reduced calcium level in BMP-4 treated MC3T3-E1 cells, while BMP-4 induced about 3-fold increase of calcium level compared to untreated control.

3.3. UCN-01 downregulated the phosphorylation of PKC βII in BMP-4-treated MC3T3-E1 cells

To examine which subfamilies of PKCs are involved in the signaling cascade of osteoblastic differentiation and mineralization, two-dimensional electrophoresis immunoblotting was also carried out. As shown in Fig. 3, BMP-4 increased the phosphorylation of PKCs compared to untreated control, while UCN-01 blocked BMP induced phosphorylation of PKC subfamilies in MC3T3-E1 cells.

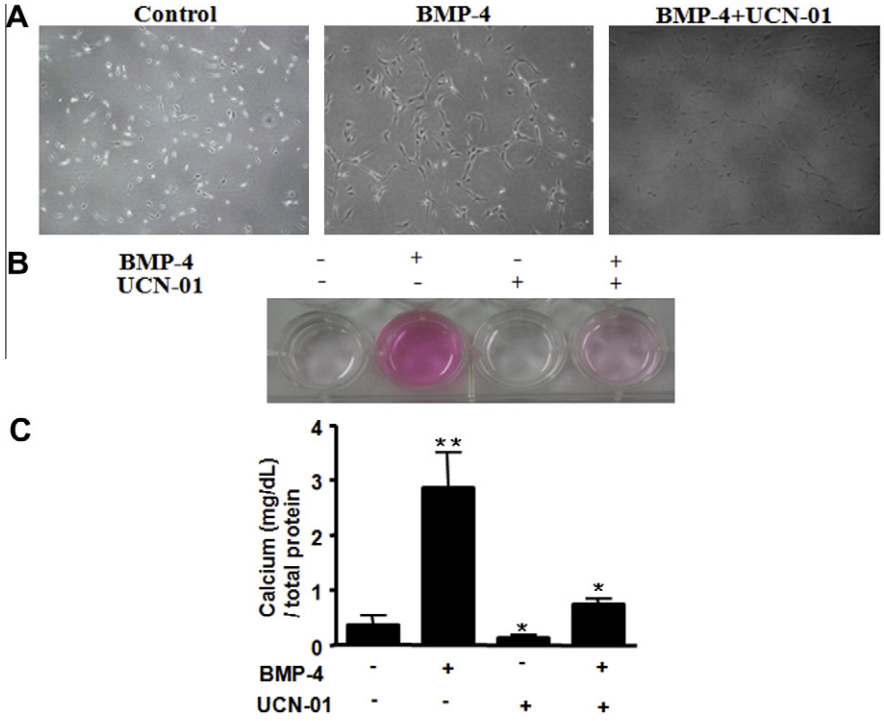

Fig. 2. UCN-01 inhibits BMP 4-induced differentiation and mineralization in MC3T3-E1 cells. Cells were seeded onto 6-well plates at a density of 2×10^5 cells/ml and stimulated with 50 ng/ml BMP-4 in the absence or presence of 25 nM UCN-01 for 10 days. (A) Differentiated cell morphology was observed under an inverted microscope. (B) ALP staining. (C) Mineralization was assessed by von Kossa staining with 3% AgNO3 for 5 min. Data represent means ± S.E., * $p < 0.05$ and **, $p < 0.01$ vs. untreated control.

Interestingly, UCN-01 downregulated the phosphorylation of PKC βII (S660), PKC α/βII (T638/641) and PKC δ/θ (S643/676) in BMP-4-treated cells. In contrast, UCN-01 did not affect the phosphorylation of PKCα, PKD/PKCμ (S744/748), PKD/PKCμ (S916) and PKC ζ/λ (T410/403) in BMP-4 treated MC3T3-E1 cells. Taken together, PKC βII and PKC δ/θ mediate BMP-4 induced differentiation in MC3T3-E1 cells.

3.4. UCN-01 reduced VEGF production in BMP-4-treated MC3T3-E1 cells

BMP-4 stimulates the synthesis of VEGF in MC3T3-E1 cells [17]. To examine whether PKC is implicated in BMP-4-mediated VEGF production, VEGF production was measured by ELISA. MC3T3-E1 cells were treated with BMP-4 and/or UCN-01 for 72 h. As shown in Fig. 4, UCN-01 significantly blocked BMP-4-induced VEGF production compared to untreated control in MC3T3-E1 cells.

4. Discussion

BMPs are known to regulate various biological actions such as embryogenesis, organogenesis, skeletogenesis, osteogenesis, cellular differentiation and apoptosis [18]. However, there is no evidence that PKCs regulate osteoblastic differentiation induced by BMP-4 until now, although PKCs are known to regulate various functions of proteins such as receptor desensitization [19], transcriptional regulation [20], and immune responses [21].

We have confirmed that BMP-4 activates ALP and osteocalcin synthesis in MC3T3-E1 cells. Regarding the correlation of BMP-2 and PKC [12], Li and colleagues reported that high glucose enhances BMP-2-induced expression of early osteogenesis genes via production of reactive oxygen species, subsequent activation of PKC, and inhibition of p38 in rat spinal ligament cells [22]. Also, Singhatanadgit and colleagues reported that TGF-β causes rapid translocation of BMPR-IB from the cytoplasm to the cell surface via PKC and p38 MAPK pathways, resulting in increased sensitivity of the cells to BMP-2 [23]. Similarly, Lemonnier and colleagues reported PKC-independent activation of protein kinase D is involved in BMP-2-induced activation of mitogen-activated protein kinase JNK and p38 and osteoblastic cell differentiation [24].

In the present study, PKC inhibitor UCN-01 significantly blocked BMP-4-mediated osteocalcin synthesis in MC3T3-E1 cells. Also, UCN-01 inhibited osteoblastic differentiation and mineralization by diluting red color for ALP staining and reducing calcium level, implying that PKC is involved in BMP-4-mediated differentiation in MC3T3-E1 cells. Interestingly, UCN-01 attenuated the phosphorylation of PKC βII (S660), PKC α/βII (T638/641) and PKC δ/θ (S643/676) in BMP-4-treated cells, while it did not affect the phosphorylation of PKC α, PKD/PKCμ (S744/748), PKD/PKCμ (S916) and PKC ζ/λ (T410/403) in BMP-4-treated MC3T3-E1 cells. Consistently, 2-DE immunoblotting revealed that UCN-01 blocked BMP-4-induced the phosphorylation of PKCs in MC3T3-E1 cells, strongly indicate that PKCs were involved in BMP-4 induced differentiation in osteo blastic MC3T3-E1 cells. Nonetheless, these findings should be confirmed by siRNA work and animal study.

Osteoblasts and osteoclasts regulate bone metabolism for bone formation, resorption [25] and remodeling. Capillary endothelial cells provide the microvasculature for the effective remodeling process [26]. In this regard, BMP-4 significantly promoted VEGF production in MC3T3-E1 cells. On the contrary, BMP-4 significantly reduced VEGF production in MC3T3-E1 cells, suggesting the involvement of PKCs in BMP-4-mediated VEGF production.

In summary, BMP-4 promoted osteocalcin synthesis and mineralization through phosphorylation of PKCs, particularly PKC βII and PKC δ/θ, in MC3T3-E1 cells. Conversely, Also, PKC inhibitor UCN-01 inhibited BMP-4-mediated osteocalcin synthesis, mineralization, VEGF production and the phosphorylation of PKCs in the cells.

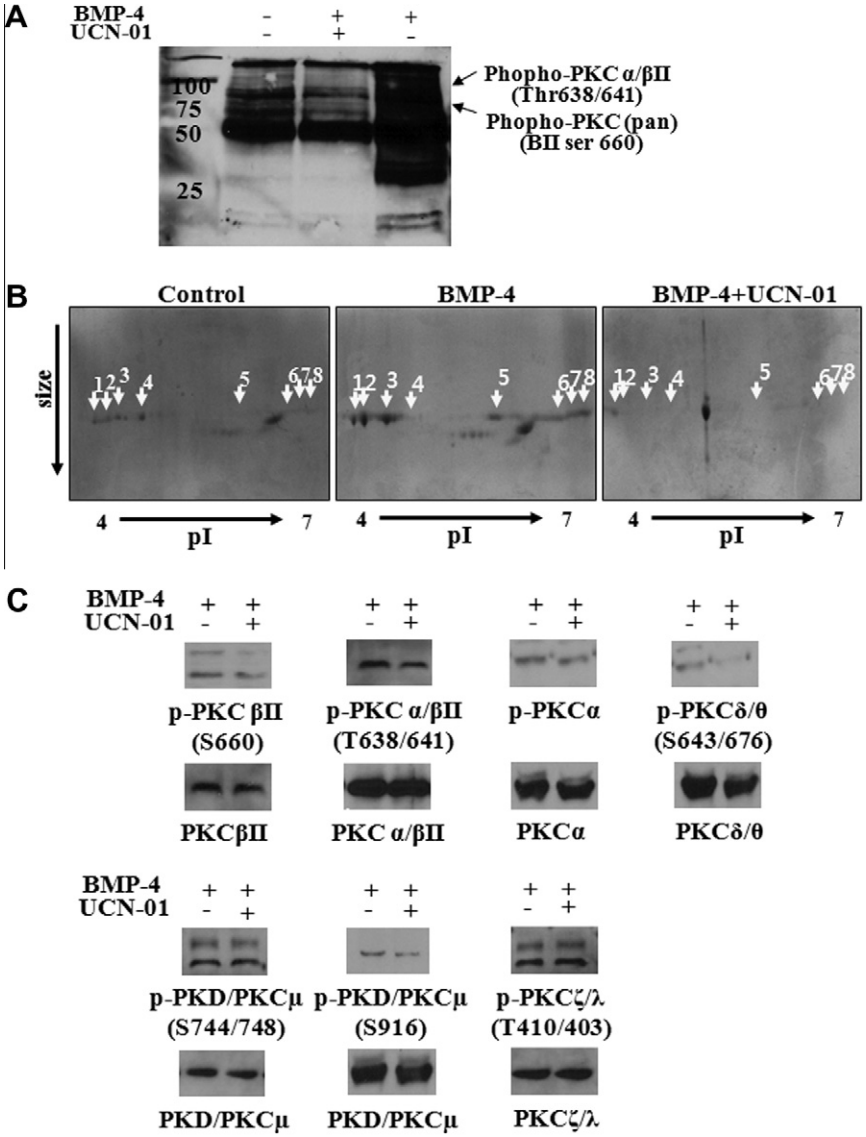

Fig. 3. UCN-01 suppresses BMP 4-induced PKC phosphorylation in MC3T3-E1 cells. Cells were pre-treated with 25 nM UCN-01 for 1 h and stimulated with or without 50 ng/ml BMP-4 for 24 h. (A) Effect of UCN-01 on the phosphorylation of PKCs by Western blotting. Cell lysates were prepared and subjected to Western blotting with anti-phospho-PKC antibodies. (B) Effect of UCN-01 on the phosphorylation of PKCs by 2-DE immunoblotting as shown in "Section 2". (C) Cells were pre-treated with 25 nM UCN-01 for 1 h and stimulated with or without 50 ng/ml BMP-4 for 24 h. Cells were subjected to Western blotting with indicated antibodies. Phospho-PKC βII (Ser 660), PKC α/βII (Thr 638/641), PKCα, PKD/PKCμ (Ser 744/748), PKC δ/θ (Ser 643/676), PKD/PKCμ (Ser 916) and PKC ζ/λ (Thr 410/403).

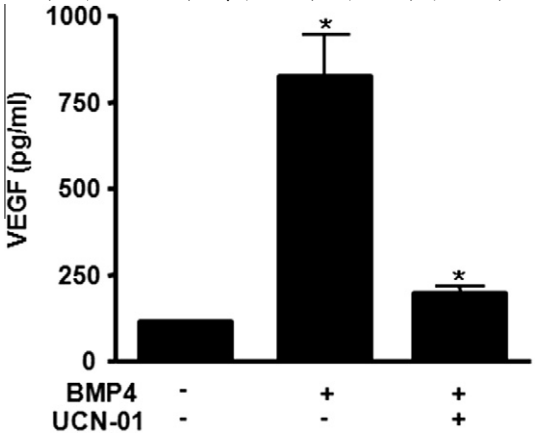

Fig. 4. UCN-01 significantly reduces VEGF production in BMP-4 mediated MC3T3-E1 cells. Cells were pre-treated with UCN-01 for 1 h and stimulated with BMP-4 for 72 h, and the supernatant was collected. The amount of VEGF was measured using mouse VEGF Quantikine M kit (R&D systems, Inc., Minneapolis, MN). Data represent means ± S.E., * $p < 0.05$ vs. untreated control.

5. Conclusion

Overall, these findings suggest that PKC βII and PKC δ/θ play important roles in BMP-4 mediated differentiation of osteoblastic cells.

Acknowledgments

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