Possible involvement of AMP-activated protein kinase in PGE_1-induced synthesis of osteoprotegerin in osteoblasts

SHINGO KAINUMA[1,2], TAKANOBU OTSUKA[1], GEN KUROYANAGI[1,2], NAOHIRO YAMAMOTO[1,2], RIE MATSUSHIMA-NISHIWAKI[2], OSAMU KOZAWA[2] and HARUHIKO TOKUDA[2,3]

[1]Department of Orthopedic Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi 467-8601; [2]Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194; [3]Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan

Received April 27, 2015; Accepted January 15, 2016

DOI: 10.3892/etm.2016.3099

Abstract. AMP-activated protein kinase (AMPK) is firmly established as a central regulator of cellular energy homeostasis. We have previously reported that prostaglandin E_1 (PGE_1) stimulates the synthesis of osteoprotegerin through p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. The present study investigated the involvement of AMPK in PGE_1-induced osteoprotegerin synthesis in MC3T3-E1 cells. The levels of osteoprotegerin were measured using an enzyme-linked immunosorbent assay, while the phosphorylation of AMPK, acetyl-CoA carboxylase, p38 MAP kinase and SAPK/JNK were analyzed by western blotting. In addition, the mRNA expression levels of osteoprotegerin were determined by a reverse transcription-quantitative polymerase chain reaction. It was revealed that PGE_1 significantly induced the phosphorylation of the α and β subunits of AMPK in a time-dependent manner ($P<0.05$). In addition, acetyl-CoA carboxylase, a direct substrate of AMPK, was significantly phosphorylated by PGE_1 ($P<0.05$). Compound C, an AMPK inhibitor, was revealed to suppress the phosphorylation of acetyl-CoA carboxylase, which significantly reduced the release and mRNA expression levels of PGE_1-stimulated osteoprotegerin ($P<0.05$). However, the PGE_1-induced phosphorylation of p38 MAP kinase and SAPK/JNK were not affected by compound C. The results of the present study indicated that AMPK may positively regulate PGE_1-stimulated osteoprotegerin synthesis in osteoblasts; thus providing novel insight into the regulatory mechanisms underlying bone metabolism.

Correspondence to: Dr Haruhiko Tokuda, Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, 7-430 Morioka, Obu, Aichi 474-8511, Japan
E-mail: tokuda@ncgg.go.jp

Key words: AMP-activated protein kinase, osteoprotegerin, prostaglandin E1, osteoblasts

Introduction

Bone metabolism is coordinated by two types of functional cells, osteoblasts and osteoclasts (1). The former cells are responsible for bone formation, whilst the latter cells are responsible for bone resorption. Thus, bone mass is sufficiently maintained by osteoclastic bone resorption followed by osteoblastic bone formation, a process termed bone remodeling (1). Disordered bone remodeling causes an imbalance in the rates of bone resorption vs. bone formation, which may result in metabolic bone diseases, including osteoporosis (1). It is at present recognized that AMP-activated protein kinase (AMPK) functions as a central regulator of cellular energy homeostasis (2,3). AMPK has been identified as an enzyme that catalyzes the phosphorylation of acetyl-CoA carboxylase, which regulates lipid synthesis (3). AMPK activity is increased by the elevation of the AMP/ATP ratio in response to physiological and pathological stress, resulting in the restoration of the cellular enzyme balance through the activation of ATP-generating pathways and the suppression of ATP-utilizing pathways (4). Therefore, evidence indicates that AMPK regulates metabolic homeostasis throughout the body (3). With respect to AMPK in bone metabolism, it has been revealed that AMPK activation stimulates bone formation and bone mass (5). We have previously demonstrated that vascular endothelial growth factor synthesis, which is induced by basic fibroblast growth factor, is positively regulated by AMPK in osteoblast-like MC3T3-E1 cells (6). Conversely, AMPK limits the interleukin (IL)-1-stimulated synthesis of IL-6 via the inhibitor of κB/nuclear factor-κB (NF-κB) signaling pathway in MC3T3-E1 cells (7). However, the roles of AMPK in osteoblasts remain undefined.

Prostaglandins are important autocrine/paracrine modulators in bone metabolism (8), and include prostaglandin E_1 (PGE_1), which has been identified as a potent bone-resorptive agent (9). It is currently accepted that osteoblasts are vital in the regulation of bone resorption through activation of receptor activator of NF-κB ligand (RANKL) (10). Osteoprotegerin is a glycoprotein secreted from osteoblasts and is a member of the tumor necrosis factor receptor superfamily, in addition to RANK (11). Osteoprotegerin binds to RANKL as a decoy receptor, thus preventing RANKL from binding to RANK. This

results in the inhibition of osteoclastogenesis and the activation of osteoclasts (11). Furthermore, RANKL knockout mice reportedly develop severe osteopetrosis (12), suggesting that RANKL is an important regulator of osteoclastogenesis. The RANK/RANKL/osteoprotegerin axis is currently recognized as a major regulatory system for the formation and activation of osteoclasts (13). Our recent study demonstrated that PGE_1 stimulates osteoprotegerin synthesis via the activation of p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), but not via the activation of p44/p42 MAP kinase, in osteoblast-like MC3T3-E1 cells (14). However, the molecular mechanism underlying the PGE_1-stimulated synthesis of osteoprotegerin in osteoblasts has yet to be elucidated.

In the present study, the involvement of AMPK in PGE_1-induced osteoprotegerin synthesis was investigated in osteoblast-like MC3T3-E1 cells. The present study revealed that AMPK acts as a positive regulator in PGE_1-stimulated osteoprotegerin synthesis in MC3T3-E1 cells.

Materials and methods

Materials. PGE_1 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and compound C was obtained from Calbiochem (cat. no. 171260; Merck Millipore, La Jolla, CA, USA). A mouse osteoprotegerin enzyme-linked immunosorbent assay (ELISA) kit (cat. no. MOP00) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Rabbit anti-phosphorylated (p)-AMPKα (Thr-172; cat. no. 2531), AMPKβ (Ser-108; cat. no. 4181), acetyl-CoA carboxylase (cat. no. 3661) polyclonal antibodies, rabbit anti-p-p38 MAP kinase (cat. no. 4511) and p-SAPK/JNK (cat. no. 4668) monoclonal antibodies, and rabbit anti-p38 MAP kinase (cat. no. 9212) and SAPK/JNK (cat. no. 9252) polyclonal antibodies, were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (sc-25778) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG; cat. no. 074-1506) was purchased from KPL, Inc. (Gaithersburg, MD, USA). An enhanced chemiluminescence western blotting detection system was purchased from GE Healthcare Life Sciences (Chalfont, UK). All other materials and chemicals were obtained from commercial sources.

PGE_1 was dissolved in ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and compound C was dissolved in dimethyl sulfoxide (Sigma-Aldrich). The maximum concentration of ethanol or dimethyl sulfoxide used was 0.1% to avoid interference with the assay for osteoprotegerin or western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria in a previous study (15) were maintained as previously described (16). Briefly, MC3T3-E1 cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes ($5x10^4$ cells/dish) or 90-mm diameter dishes ($2x10^5$ cells/dish) in α-MEM supplemented with 10% FBS. After 5 days, the medium was exchanged for α-MEM supplemented with 0.3% FBS. The cells were used for experiments following a 48-h incubation period at 37˚C.

Assay for osteoprotegerin. The cultured cells were pretreated with 0.1, 1, 3 or 10 μM compound C for 60 min. The cells were stimulated with 10 μM PGE_1 or vehicle [phosphate-buffered saline (PBS) supplemented with 0.01% bovine serum albumin (Sigma-Aldrich) containing 0.1% ethanol] in 1 ml α-MEM supplemented with 0.3% FBS, and then incubated for 48 h. The conditioned medium was collected by aspiration subsequent to incubation, followed by measurement with a mouse osteoprotegerin ELISA kit, according to the manufacturer's protocol.

Western blot analysis. The cultured cells were pretreated with 0.1, 1, 3 or 10 μM of compound C for 60 min, and then stimulated with 10 μM PGE_1 or vehicle in α-MEM supplemented with 0.3% FBS for 0, 1, 3, 5, 10, 20, 30 or 60 min. The cells were washed twice with PBS and then lysed, homogenized and sonicated in 900 μl lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. Protein samples were obtained from the lysed cells using a cell scraper, after which equal quantities of protein (10 μl) were separated by SDS-polyacrylamide gel electrophoresis, according to a previous study (17), on 10% polyacrylamide gels. The electrophoresis was run at 100 V for the separating gel and 250 V for the stacking gel, until the dye front ran off the bottom of the gel. The protein was fractionated and transferred to an Immun-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was performed as previously described (18). Briefly, the membranes were blocked with 5% fat-free dry milk in Tris-buffered saline containing Tween-20 (TBST; 20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 h prior to incubation with the primary antibodies. The membranes were incubated overnight at 4˚C with the rabbit anti-p-AMPK, anti-acetyl-CoA carboxylase, anti-GAPDH, anti-p-p38 MAP kinase, anti-p38 MAP kinase, anti-p-SAPK/JNK and anti-SAPK/JNK primary antibodies at a dilution of 1:1,000 in 5% milk containing TBST. Subsequently, the membranes were washed three times for 5 min each with TBST, and then incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:1,000 in 5% milk containing TBST. Peroxidase activity on the PVDF membrane was visualized on X-ray film (Super RX; FUJIFILM, Kanagawa, Japan) by means of the enhanced chemiluminescence western blotting detection system.

Densitometric analysis. Densitometric analysis of the western blots was performed using a scanner (GT-F600; Seiko Epson Corporation, Nagano, Japan) and Image J image analysis software program (version 1.48; National Institutes of Health, Bethesda, MD, USA). The background-subtracted signal intensity for each phosphorylation signal was normalized to the respective total protein signal, and plotted as the fold increase in comparison to control cells that did not receive stimulation.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cultured cells were pretreated with 10 μM compound C or vehicle for 60 min, and then stimulated with

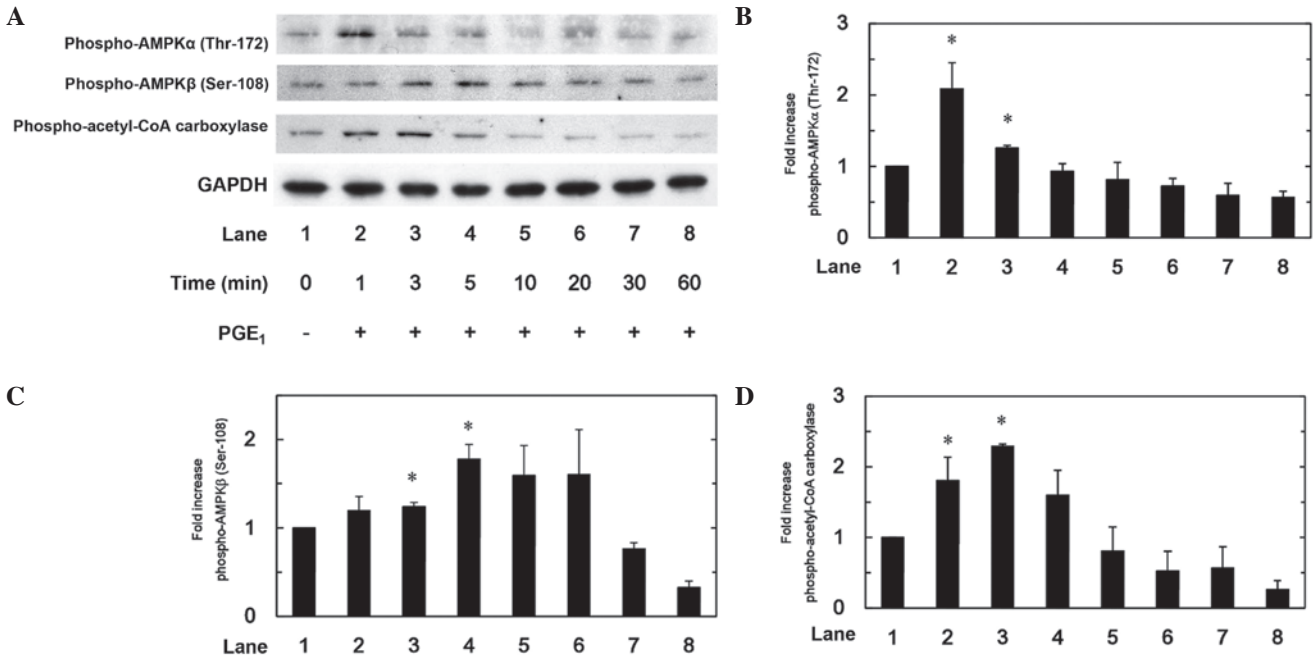

Figure 1. Effects of PGE_1 on the phosphorylation of AMPK or acetyl-CoA carboxylase in MC3T3-E1 cells. The cultured cells were stimulated by 10 μM PGE_1 for the indicated periods. (A) Cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent western blot analysis. Histograms display the quantified expression levels of PGE_1-induced phosphorylation of (B) AMPKα (Thr-172), (C) AMPKβ (Ser-108) and (D) acetyl-CoA carboxylase, as determined by laser densitometric analysis. The phosphorylation levels were corrected against the GAPDH expression levels and expressed as fold increase compared with the basal levels presented in lane 1. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations. *P<0.05 vs. control. AMPK, adenosine monophosphate-activated protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGE_1, prostaglandin E_1.

10 μM of PGE_1 or vehicle in α-MEM supplemented with 0.3% FBS for 3 h. Total RNA was isolated and reverse transcribed into cDNA using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and an Omniscript Reverse Transcriptase kit (Qiagen, Inc., Valencia, CA, USA), respectively. RT-qPCR was performed in capillaries using a LightCycler® 480 system with the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Basel, Switzerland) and 1 ml cDNA. The primer sequences were as follows: Osteoprotegerin forward, 5'-CAATGGCTGGCTTGGTTTCATAG-3' and reverse, 5'-CTGAACCAGACATGACAGCTGGA-3' (Takara Bio, Inc., Otsu, Japan); and GAPDH forward, 5'-AACGACCCCTTC ATTGAC-3' and reverse, 5'-TCCACGACATACTCAGCAC-3' (Sigma-Aldrich). The reaction mixtures were incubated at 95˚C for 10 min, followed by 40 cycles at 60˚C for 5 sec and 72˚C for 7 sec. The amplified products were confirmed by a melting curve analysis, according to the system protocol. The osteoprotegerin mRNA expression levels were normalized to those of GAPDH mRNA using PASW Statistics software, version 18 (SPSS, Inc., Chicago, IL, USA) and the relative mRNA expression levels were determined from the basis of a standard curve, which was created automatically with the LightCycler software in each run. The results are the average of three independent experiments.

Statistical analysis. The data were analyzed by analysis of variance followed by the Bonferroni method for multiple comparisons between pairs. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were conducted using Microsoft Office Excel 2013 for Windows (Microsoft, Redmond, WA, USA) Data are presented as the means ± standard error of the mean of triplicate determinations from three independent cell preparations.

Results

PGE_1 induces the phosphorylation of AMPK and acetyl-CoA carboxylase in MC3T3-E1 cells. It is firmly established that the phosphorylation of AMPK is necessary for its activation (19). Therefore, to determine whether AMPK is activated by PGE_1 in osteoblast-like MC3T3-E1 cells, the present study initially examined the effect of PGE_1 on the phosphorylation of AMPK (Fig. 1A). PGE_1 significantly induced the phosphorylation of AMPKα (Thr-172; P<0.05; Fig. 1B) and AMPKβ (Ser-108; P<0.05; Fig. 1C). The effects of PGE_1 on the phosphorylation of AMPKα and AMPKβ reached peak levels at 1 and 5 min after stimulation, respectively; and decreased thereafter. Subsequent to this, as acetyl-CoA carboxylase is a direct substrate of AMPK (3), the effect of PGE_1 on the phosphorylation of acetyl-CoA carboxylase in MC3T3-E1 cells was examined. PGE_1 significantly stimulated the phosphorylation of acetyl-CoA carboxylase, displaying peak levels 3 min after stimulation (Fig. 1D).

Compound C suppresses PGE_1-stimulated osteoprotegerin release in MC3T3-E1 cells. Our previous study recently revealed that PGE_1 stimulates osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells (14). In order to investigate the involvement of AMPK in PGE_1-induced synthesis of osteoprotegerin in the aforementioned cells, the effect of compound C,

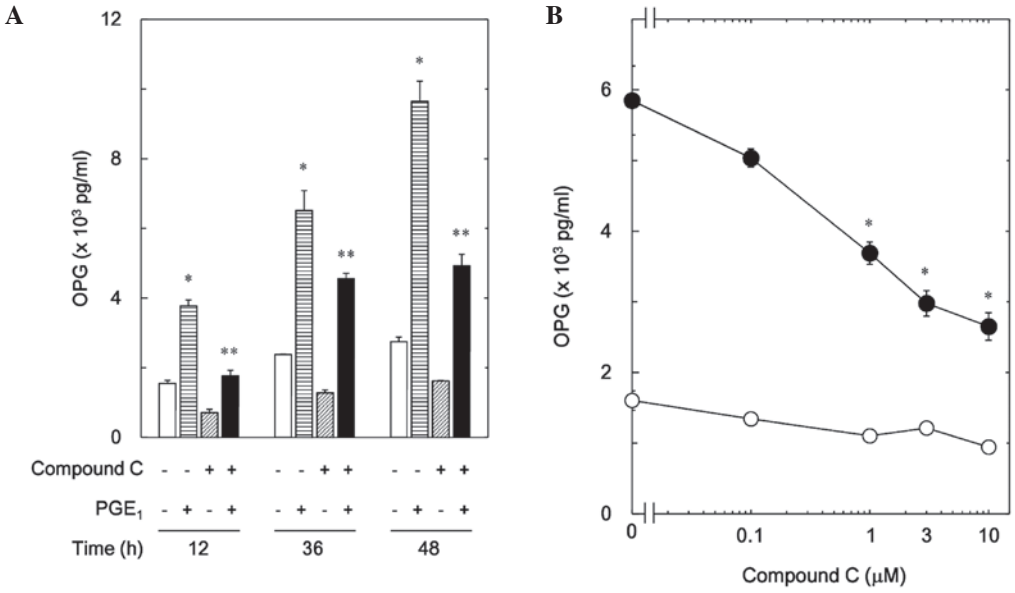

Figure 2. Effect of compound C on PGE_1-stimulated OPG release in MC3T3-E1 cells. (A) Cultured cells were pretreated with 10 μM compound C or vehicle for 60 min, and then stimulated with 10 μM of PGE_1 or vehicle for the indicated periods. The OPG concentrations of the culture medium were determined by ELISA. *P<0.05 vs. control; **P<0.05 vs. PGE_1 alone. (B) Cells were pretreated with various concentrations of compound C for 60 min, and then stimulated with 10 μM of PGE_1 (●) or vehicle (○) for 48 h. The OPG concentrations of the culture medium were determined by ELISA. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations. *P<0.05 vs. PGE_1 alone. OPG, osteoprotegerin; PGE_1, prostaglandin E_1.

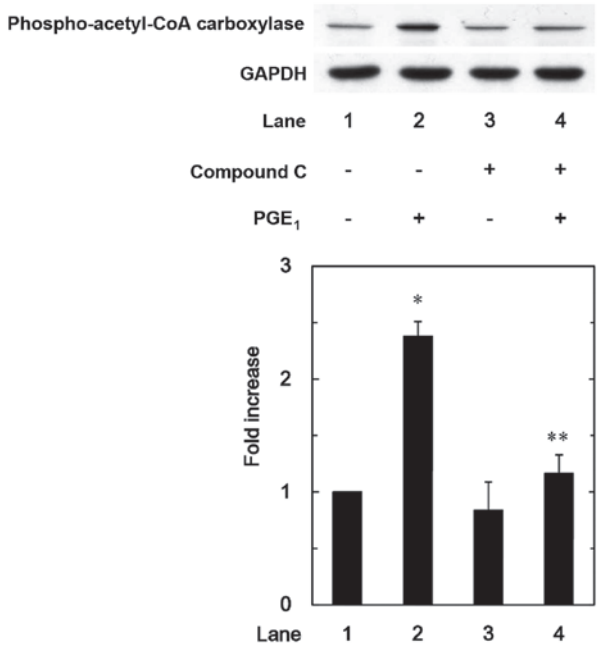

Figure 3. Effect of compound C on PGE_1-stimulated phosphorylation of acetyl-CoA carboxylase in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM compound C or vehicle for 60 min, and then stimulated with 10 μM of PGE_1 or vehicle for 3 min. The extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent western blot analysis using phospho-specific acetyl-CoA carboxylase antibodies or GAPDH. The histogram indicates the quantification of the levels of the PGE_1-induced phosphorylation obtained from laser densiometric analysis of three independent experiments. The phosphorylation levels were corrected by the GAPDH expression levels, and are presented as the fold increase with respect to the basal levels presented in lane 1. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations. *P<0.05 vs. control; **P<0.05 vs. PGE_1 alone. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGE_1, prostaglandin E_1.

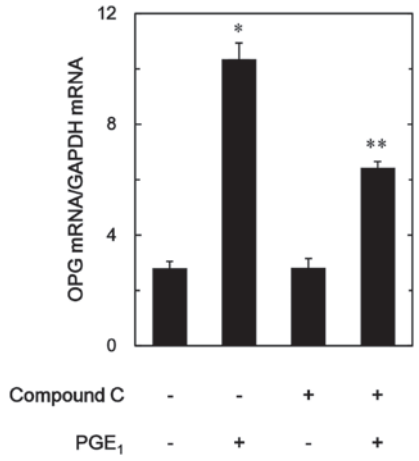

Figure 4. Effect of compound C on PGE_1-induced OPG mRNA expression in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM compound C or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 3 h. The respective total RNA was then isolated and quantified by reverse transcription-quantitative polymerase chain reaction. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations. *P<0.05 vs. control; **P<0.05 vs. PGE_1 alone. OPG, osteoprotegerin; PGE_1, prostaglandin E_1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

an AMPK inhibitor (20), on PGE_1-stimulated release of osteoprotegerin was examined. Compound C significantly suppressed PGE_1-stimulated osteoprotegerin release compared with cells treated with PGE_1 alone (P<0.05; Fig. 2A). The inhibitory effect of compound C was dose-dependent between 0.1 and 10 mM (Fig. 2B). The maximum suppressive activity of compound C was observed at 10 μM, producing 60% inhibition of the effects of PGE_1.

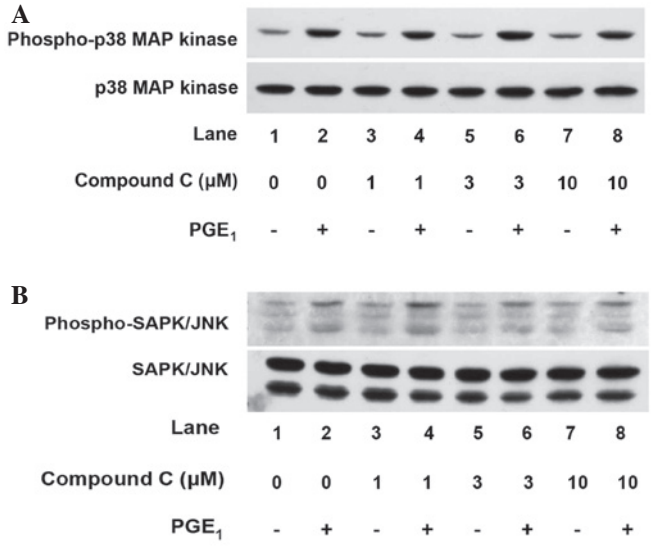

Figure 5. Effects of compound C on PGE_1-stimulated phosphorylation levels of (A) p38 MAP kinase and (B) SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various concentrations of compound C for 60 min. The cells were then stimulated with 10 μM PGE_1 or vehicle for (A) 10 or (B) 20 min, respectively. The extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent western blot analysis using antibodies against phospho-p38 MAP kinase, p38 MAP kinase, phospho-SAPK/JNK or SAPK/JNK. MAP, mitogen-activated protein; PGE_1, prostaglandin E_1; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase.

Additionally, the effect of compound C on PGE_1-induced phosphorylation of acetyl-CoA carboxylase in MC3T3-E1 cells was examined. PGE_1-induced phosphorylation of acetyl-CoA carboxylase was significantly reduced by compound C compared with PGE_1 treatment alone ($P<0.05$; Fig. 3).

Compound C suppresses PGE_1-induced osteoprotegerin mRNA expression levels in MC3T3-E1 cells. To determine whether the inhibition of the PGE_1-stimulated osteoprotegerin release by compound C is mediated through transcriptional events in osteoblast-like MC3T3-E1 cells, the effect of compound C on PGE_1-induced osteoprotegerin mRNA expression levels were examined by RT-qPCR. The expression levels of osteoprotegerin mRNA induced by PGE_1 were significantly suppressed by compound C compared with PGE_1 treatment alone ($P<0.05$; Fig. 4).

Compound C does not affect PGE_1-stimulated phosphorylation of p38 MAP kinase or SAPK/JNK in MC3T3-E1 cells. Regarding the intracellular signaling of PGE_1 in osteoblasts, our previous study revealed that PGE_1 induces the activation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells. In addition, p38 MAP kinase and SAPK/JNK were implicated in PGE_1-stimulated osteoprotegerin synthesis, whilst p44/p42 MAP kinase was not (14). To investigate whether the effect of AMPK on PGE_1-stimulated osteoprotegerin synthesis is associated with the activation of p38 MAP kinase or SAPK/JNK in MC3T3-E1 cells, the effects of compound C on PGE_1-induced phosphorylation of p38 MAP kinase and SAPK/JNK were examined. However, compound C did not affect PGE_1-induced phosphorylation of p38 MAP kinase (Fig. 5A) or SAPK/JNK (Fig. 5B) between 1 and 10 μM.

Discussion

The present study revealed that PGE_1 significantly stimulates the phosphorylation of AMPKα (Thr-172) and AMPKβ (Ser-108) in osteoblast-like MC3T3-E1 cells. The heterotrimeric AMPK complex is comprised of α, β and γ subunits (2). Of the aforementioned subunits of AMPK, the α subunit is a catalytic subunit. Conversely, the β and γ subunits are regulatory subunits (19). It is currently recognized that the phosphorylation of Thr-172, located within the α subunit, is required for AMPK activity, while phosphorylation of the β subunit is necessary for the activation of AMPK (19). The present study revealed that the phosphorylation of acetyl-CoA carboxylase was significantly induced by PGE_1 in MC3T3-E1 cells. It is generally accepted that activated AMPK induces the phosphorylation of acetyl-CoA carboxylase, a direct substrate of AMPK, resulting in the stimulation of the oxidation of fatty acid as a result of its inhibition (3). The maximum effect of PGE_1 on the phosphorylation of acetyl-CoA carboxylase was observed 3 min after stimulation. Comparatively, the α subunit demonstrated peak phosphorylation levels within 1 min. Thus, the time course of AMPKα phosphorylation appeared to be more rapid than that of acetyl-CoA carboxylase. As a result, PGE_1-induced activation of acetyl-CoA carboxylase occurs subsequent to AMPKα activation. Considering the findings of the present study, it is possible that PGE_1 induces AMPK activation in osteoblast-like MC3T3-E1 cells.

Our previous study recently reported that PGE_1 stimulates osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells (14). The involvement of AMPK in osteoprotegerin synthesis has since been investigated, and it was revealed that the PGE_1-stimulated release of osteoprotegerin was significantly suppressed by compound C in MC3T3-E1 cells (20). In agreement, the present study revealed that compound C was able to inhibit PGE_1-induced phosphorylation of acetyl-CoA carboxylase in these cells. Therefore, it is possible that PGE_1-activated AMPK is involved in osteoprotegerin release. Additionally, the present study demonstrated that PGE_1-induced mRNA expression levels of osteoprotegerin were significantly downregulated by compound C, suggesting that the effect of compound C is exerted through inhibition of the transcriptional process. Based on the findings of the present study, it is proposed that PGE_1 stimulates AMPK activation, resulting in the induction of osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells.

With regard to the intracellular signaling of PGE_1 in osteoblasts, our previous study demonstrated that PGE_1 induces the activation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (21,22), and that p38 MAP kinase and SAPK/JNK (but not p44/p42 MAP kinase) are involved in PGE_1-stimulated osteoprotegerin synthesis (14). The MAP kinase superfamily have a crucial role in various cellular functions, including proliferation, differentiation and survival (23). It is generally recognized that p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK are three major MAP kinases necessary for the transduction of diverse cellular messages (24). In order to investigate the molecular

mechanism employed by AMPK in PGE_1-stimulated osteoprotegerin synthesis in MC3T3-E1 cells, the association between AMPK and the aforementioned MAP kinases were investigated. However, the present study revealed that compound C failed to effect the PGE_1-induced phosphorylation of p38 MAP kinase or SAPK/JNK. Therefore, it is likely that AMPK may act downstream of the aforementioned MAP kinases, or through a different target located upstream of transcription, resulting in osteoprotegerin synthesis. Further investigation is required to identify the exact mechanism by which AMPK is involved in PGE_1-stimulated osteoprotegerin synthesis in osteoblasts.

It is currently established that AMPK has a central role in energy homeostasis, particularly in myocytes and adipocytes (2). Regarding the roles of AMPK in osteoblasts, it has been revealed that the activation of AMPK stimulates collagen synthesis and the induction of Runt-related transcription factor 2 expression, and enhances mineralization (25,26). Additionally, AMPK has been reported to have an inhibitory role in palmitate-induced apoptosis (27). Thus, accumulating evidence suggests that AMPK activation in osteoblasts may stimulate them toward differentiation. It is accepted that PGE_1 acts as an autocrine/paracrine regulator of osteoblasts and also regulates bone remodeling (8). Conversely, it is established that osteoprotegerin acts as a decoy receptor to prevent RANKL from binding RANK in osteoclast progenitor cells, resulting in the downregulation of osteoclastogenesis and bone resorption (11). Based on the findings of the present study, it is proposed that AMPK activated by PGE_1 in osteoblasts directs the bone remodeling process to favor formation rather than resorption by stimulating osteoprotegerin synthesis. Further investigation is necessary to elucidate the exact roles of AMPK in bone metabolism.

In conclusion, the results of the present study strongly suggest that AMPK positively regulates the PGE_1-stimulated synthesis of osteoprotegerin in osteoblasts. The present study may provide novel insights into the regulatory mechanisms underlying bone metabolism.

Acknowledgements

The present authors are grateful to Mrs. Yumiko Kurokawa for her skillful technical assistance. The present study was supported in part by a Grant-in-Aid for Scientific Research (grant no. 19591042) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Grant-in-Aid for Scientific Research (grant no. H25-Aging-General-004) from the Ministry of Health, Labour and Welfare of Japan; and the Research Funding for Longevity Sciences (grant no. 25-4, 26-12) from the National Center for Geriatrics and Gerontology (Obu, Japan).

References

1. Karsenty G and Wagner EF: Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2: 389-406, 2002.
2. Fogarty S and Hardie DG: Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. Biochim Biophys Acta 1804: 581-591, 2010.
3. Mihaylova MM and Shaw RJ: The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat Cell Biol 13: 1016-1023, 2011.
4. Rutter GA and Leclerc I: The AMP-regulated kinase family: Enigmatic targets for diabetes therapy. Mol Cell Endocrinol 297: 41-49, 2009.
5. Shah M, Kola B, Bataveljic A, Arnett TR, Viollet B, Saxon L, Korbonits M and Chenu C: AMP-activated protein kinase (AMPK) activation regulates in vitro bone formation and bone mass. Bone 47: 309-319, 2010.
6. Kato K, Tokuda H, Adachi S, Matsushima-Nishiwaki R, Natsume H, Yamakawa K, Gu Y, Otsuka T and Kozawa O: AMP-activated protein kinase positively regulates FGF-2-stimulated VEGF synthesis in osteoblasts. Biochem Biophys Res Commun 400: 123-127, 2010.
7. Kato K, Tokuda H, Matsushima-Nishiwaki R, Natsume H, Kondo A, Ito Y, Kozawa O and Otsuka T: AMPK limits IL-1-stimulated IL-6 synthesis in osteoblasts: Involvement of IκB/NF-κB pathway. Cell Signal 24: 1706-1712, 2012.
8. Hikiji H, Takato T, Shimizu T and Ishii S: The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease. Prog Lipid Res 47: 107-126, 2008.
9. Zhu Z, Fu C, Li X, Song Y, Li C, Zou M, Guan Y and Zhu Y: Prostaglandin E2 promotes endothelial differentiation from bone marrow-derived cells through AMPK activation. PLoS One 6: e23554, 2011.
10. Boyce BF and Xing L: Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys 473: 139-146, 2008.
11. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T *et al*: Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 89: 309-319, 1997.
12. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C Morony S, Oliveira-dos-Santos AJ, Van G, Itie A *et al*: OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397: 315-323, 1999.
13. Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F and Heymann D: The molecular triad OPG/RANK/RANKL: Involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 15: 457-475, 2004.
14. Yamamoto N, Otsuka T, Kuroyanagi G, Kondo A, Kainuma S, Nakakami A, Matsushima-Nishiwaki R, Kozawa O and Tokuda H: Resveratrol reduces prostaglandin E1-stimulated osteoprotegerin synthesis in osteoblasts: Suppression of stress-activated protein kinase/c-Jun N-terminal kinase. Prostaglandins Other Lipid M Mediat 116-117: 57-63, 2015.
15. Sudo H, Kodama HA, Amagai Y, Yamamoto S and Kasai S: In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96: 191-198, 1983.
16. Kozawa O, Tokuda H, Miwa M, Kotoyori J and Oiso Y: Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E2 in osteoblast-like cells. Exp Cell Res 198: 130-134, 1992.
17. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
18. Kato K, Ito H, Hasegawa K, Inaguma Y, Kozawa O and Asano T: Modulation of the stress-induced synthesis of hsp27 and alpha B-crystallin by cyclic AMP in C6 rat glioma cells. J Neurochem 66: 946-950, 1996.
19. Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D and Hardie DG: Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J Biol Chem 271: 27879-27887, 1996.
20. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N *et al*: Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 108: 1167-1174, 2001.
21. Tokuda H, Kozawa O, Miwa M and Uematsu T: p38 mitogen-activated protein (MAP) kinase but not p44/p42 MAP kinase is involved in prostaglandin E1-induced vascular endothelial growth factor synthesis in osteoblasts. J Endocrinol 170: 629-638, 2001.
22. Kanno Y, Tokuda H, Nakajima K, Ishisaki A, Shibata T, Numata O and Kozawa O: Involvement of SAPK/JNK in prostaglandin E(1)-induced VEGF synthesis in osteoblast-like cells. Mol Cell Endocrinol 220: 89-95, 2004.
23. Kyriakis JM and Avruch J: Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81: 807-869, 2001.

24. Widmann C, Gibson S, Jarpe MB and Johnson GL: Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiol Rev 79: 143-180, 1999.
25. Kanazawa I, Yamaguchi T, Yano S, Yamauchi M and Sugimoto T: Metformin enhances the differentiation and mineralization of osteoblastic MC3T3-E1 cells via AMP kinase activation as well as eNOS and BMP-2 expression. Biochem Biophys Res Commun 375: 414-419, 2008.
26. Jang WG, Kim EJ, Lee KN, Son HJ and Koh JT: AMP-activated protein kinase (AMPK) positively regulates osteoblast differentiation via induction of Dlx5-dependent Runx2 expression in MC3T3E1 cells. Biochem Biophys Res Commun 404: 1004-1009, 2011.
27. Kim JE, Ahn MW, Baek SH, Lee IK, Kim YW, Kim JY, Dan JM and Park SY: AMPK activator, AICAR, inhibits palmitate-induced apoptosis in osteoblast. Bone 43: 394-404, 2008.