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AMP-activated protein kinase positively regulates FGF-2-stimulated VEGF synthesis in osteoblasts

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ABSTRACT

AMP-activated protein kinase (AMPK) is recognized as a regulator of energy homeostasis. We have previously reported that basic fibroblast growth factor (FGF-2) stimulates vascular endothelial growth factor (VEGF) release through the activation of p44/p42 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the involvement of AMPK in FGF-2-stimulated VEGF release in these cells. FGF-2 time-dependently induced the phosphorylation of AMPK α-subunit (Thr-172). Compound C, an AMPK inhibitor, which suppressed the FGF-2-induced phosphorylation of AMPK, significantly inhibited the VEGF release stimulated by FGF-2. The AMPK inhibitor also reduced the mRNA expression of VEGF induced by FGF-2. The FGF-2-induced phosphorylation of both p44/p42 MAP kinase and SAPK/JNK was attenuated by compound C. These results strongly suggest that AMPK positively regulates the FGF-2-stimulated VEGF synthesis via p44/p42 MAP kinase and SAPK/JNK in osteoblasts.

1. Introduction

AMP-activated protein kinase (AMPK), discovered in 1973, has been recognized at first as an emergency response enzyme which would be activated only during severe metabolic stress [1,2]. AMPK is currently known to regulate gene expression or control whole body metabolic homeostasis, and several biosynthetic enzymes for fatty acid and glycogen are recognized as the targets of AMPK [3]. AMPK consists of three subunits, named α, β, and γ [4,5]. α-Subunit is considered as a catalytic site, while β and γ-subunits are as regulatory sites [6]. The phosphorylation of Thr-172 in the α-subunit is essential for AMPK activation [5]. AMPK is phosphorylated by elevation of the AMP:ATP ratio, increase of cytosolic free Ca^{2+}, and transforming growth factor-1β-activated kinase (TAK1) [3]. It is well recognized that the activation of AMPK results in suppression of glucose-induced insulin secretion and decrease of cell survival in pancreatic β-cells [3]. It has been reported that the activated AMPK leads to the recruitment to plasma membrane of the glucose transporter Glut4 in skeletal muscle, which causes up-taking of glucose into cells [7]. In ovarian cancer cells, the inhibition of fatty acid synthase reportedly activates AMPK, resulting in causing cytotoxicity [8]. Therefore, AMPK is proposed as a new therapeutic target for diabetes, cancer, and obesity.

Bone metabolism is highly coordinated process of bone resorption by osteoclasts and bone formation by osteoblasts [9]. As for the relationship between bone metabolism and AMPK, it has been reported that metformin, an activator of AMPK, increases collagen-1 and osteocalcin mRNA expression, stimulates alkaline phosphatase activity, and enhances cell mineralization in osteoblast-like MC3T3-E1 cells [10]. It has recently been shown that AMPK activation regulates bone formation and bone mass [11]. These previous findings lead us to speculate that AMPK affects bone metabolism through the functional modulation of osteoblasts. However, the exact role of AMPK in bone metabolism, especially in osteoblasts, has not yet been fully clarified.

It is recognized that osteoblasts synthesize basic fibroblast growth factor (FGF-2), and FGF-2 is embedded in bone matrix [12,13]. During fracture repairing, FGF-2 expression is detected in osteoblasts [14]. Accumulating evidence suggests that FGF-2 plays a pivotal role in fracture healing, bone remodeling, and osteogenesis. There are four receptors with high structural affinity (FGF receptors 1–4) [15]. We have previously reported that FGF-2 activates FGF receptors 1 and 2 among those four receptors in osteoblast-like MC3T3-E1 cells [16]. We have also reported that FGF-2 stimulates the release of vascular endothelial growth factor (VEGF), a mitogen highly specific for vascular endothelium responding to various physiological stimulants such as insulin-like growth factor-1 and bone morphogenetic protein [17]. VEGF is recognized to play an important role in bone remodeling to provide

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microvasculature [18]. Regarding the signaling mechanism in osteoblasts, we have reported that VEGF release stimulated by FGF-2 is positively regulated by p44/p42 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) [19,20], among the MAP kinase superfamily [21]. Based on these findings, VEGF secreted from osteoblasts may couple angiogenesis to bone formation by adjusting the angiogenic response to osteoblastic activity. However, the exact mechanism behind VEGF synthesis in osteoblasts and its release from these cells is not precisely elucidated.

In the present study, we investigated the role of AMPK in FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. We here show that AMPK positively regulates FGF-2-stimulated VEGF synthesis through the activation of p44/p42 MAP kinase and SAPK/JNK in osteoblasts.

2. Materials and methods

2.1. Materials

Mouse VEGF enzyme-linked immunosorbent assay (ELISA) kits and FGF-2 were purchased from R&D Systems, Inc. (Minneapolis, MN). Compound C was purchased from Calbiochem–Novabiochem (La Jolla, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-specific AMPK α (Thr-172) antibodies, phospho-specific AMPK α (Ser-485) antibodies, phospho-specific AMPK β (Ser-108) antibodies, phospho-specific AMPK β (Ser-182) antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). ECL Western blotting detection system was obtained from GE Healthcare UK Ltd. (Buckinghamshire, UK). Trizol reagent and omniscript reverse transcriptase kit were purchased from Invitrogen (Carlsbad, CA) and QIAGEN (Hilden, Germany), respectively. Fast-start DNA Master SYBR Green I was purchased from Roche Diagnostics (Mannheim, Germany). Other materials and chemicals were obtained from commercial sources.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [22], were maintained as previously described [23]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35- or 90-mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged to α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. VEGF assay

The cultured cells were stimulated by 30 ng/ml FGF-2 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with various doses of compound C for 60 min. The conditioned medium was collected at the end of the incubation, and the VEGF concentration was measured by VEGF ELISA kit.

2.4. Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm and 560 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT).

2.5. Western blot analysis

The cultured cells were stimulated by 30 ng/ml FGF-2 in α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with various doses of compound C for 60 min. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS–polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [24] in 10% polyacrylamide gel. Western blotting analysis was performed as previously described [25] by using phospho-specific AMPK α (Thr-172) antibodies, phospho-specific AMPK α (Ser-485) antibodies, phospho-specific AMPK β (Ser-108) antibodies, phospho-specific AMPK β (Ser-182) antibodies, GAPDH antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

2.6. Real-time RT-PCR

The cultured cells were pretreated with compound C for 60 min, and stimulated by 30 ng/ml FGF-2 for the indicated period. Total RNA was isolated and transcribed into cDNA using Trizol reagent and Omniscript Reverse Transcriptase Kit. Real-time PCR was performed using a Light Cycler system (Roche Diagnostics) in capillaries and Fast-Start DNA Master SYBR Green I provided with the kit.

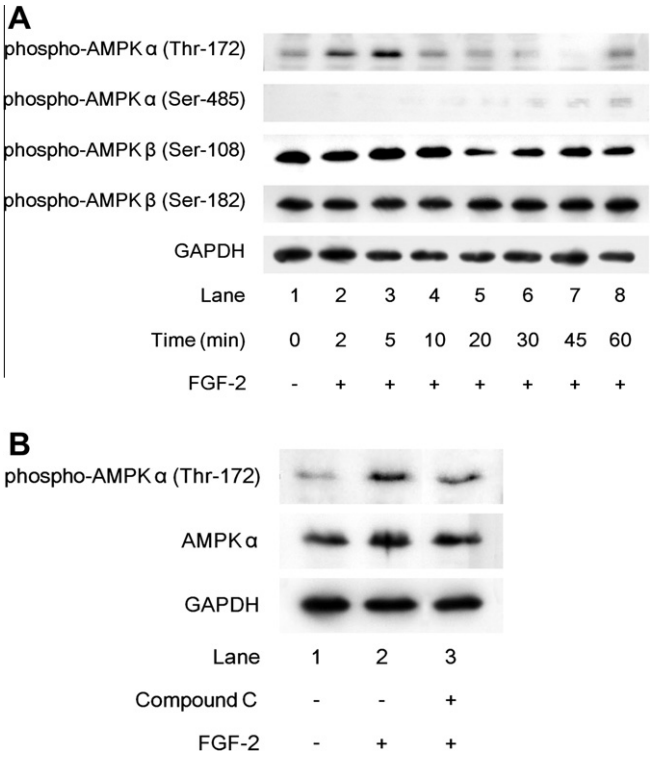

Fig. 1. Effects of FGF-2 on the phosphorylation of AMPK in MC3T3-E1 cells. (A) The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. (B) The cultured cells were pretreated with 10 μM AMPK inhibitor or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 for 5 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific AMPK α-subunit (Thr-172), phospho-specific AMPK α-subunit (Ser-485), phospho-specific AMPK β-subunit (Ser-108), phospho-specific AMPK β-subunit (Ser-182), GAPDH or AMPK α-subunit.

Sense and antisense primers were synthesized based on the report of Simpson et al. [26] for mouse VEGF mRNA and GAPDH mRNA. The amplified products were determined by melting curve analysis and agarose electrophoresis. VEGF mRNA levels were normalized with those of GAPDH mRNA.

2.7. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $p < 0.05$ was considered significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effects of FGF-2 on the phosphorylation of AMPK in MC3T3-E1 cells

In order to clarify whether FGF-2 activates AMPK in osteoblasts, we first examined the effects of FGF-2 on the phosphorylation of AMPK in osteoblast-like MC3T3-E1 cells. FGF-2 remarkably induced the phosphorylation of AMPK α-subunit (Thr-172). The effect of FGF-2 on the phosphorylation of AMPK reached its peak at 5 min and decreased thereafter (Fig. 1A). However, FGF-2 did not affect the phosphorylation of α-subunit (Ser-485) (Fig. 1A). On the other hand, AMPK β-subunit (Ser-108 and Ser-182) was phosphorylated even in unstimulated MC3T3-E1 cells and FGF-2 had little effects on the phosphorylation (Fig. 1A). In addition, we found that compound C, an inhibitor of AMPK, truly suppressed the FGF-2-induced phosphorylation levels of AMPK α-subunit (Fig. 1B. Lane 3 compared to Lane 2).

3.2. Effect of AMPK inhibitor on the FGF-2-stimulated VEGF release in MC3T3-E1 cells

In order to explore the involvement of AMPK in the FGF-2-induced VEGF release in osteoblasts, we next examined the effect of compound C on the release of VEGF induced by FGF-2 in MC3T3-E1 cells. Compound C, which alone had little effect on the VEGF levels, significantly suppressed the FGF-2-induced VEGF release (Fig. 2). The inhibitory effect of compound C was dose-dependent in the range between 0.1 and 10 μM. The maximum effect of compound C was observed at 10 μM, caused approximately 60% inhibition in the FGF-2 effect.

3.3. Effects of AMPK inhibitor on the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells

We previously reported that FGF-2 stimulates VEGF release via p44/p42 MAP kinase and SAPK/JNK among the MAP kinase superfamily in osteoblast-like MC3T3-E1 cells [19,20]. In order to investigate whether the effect of AMPK on the FGF-2-stimulated VEGF synthesis is related to the activation of p44/p42 MAP kinase, we examined the effect of compound C on the phosphorylation of p44/p42 MAP kinase by FGF-2. AMPK inhibitor markedly attenuated the FGF-2-induced phosphorylation of p44/p42 MAP kinase (Fig. 3A).

We next examined the effect of compound C on the phosphorylation of SAPK/JNK in MC3T3-E1 cells. In addition to p44/p42 MAP kinase, compound C (10 μM) remarkably suppressed the FGF-2-induced phosphorylation of SAPK/JNK (Fig. 3B).

3.4. Effect of AMPK inhibitor on the FGF-2-induced VEGF mRNA in MC3T3-E1 cells

It has been shown that FGF-2 induces the expression of VEGF in osteoblasts [27]. In order to elucidate investigate whether the

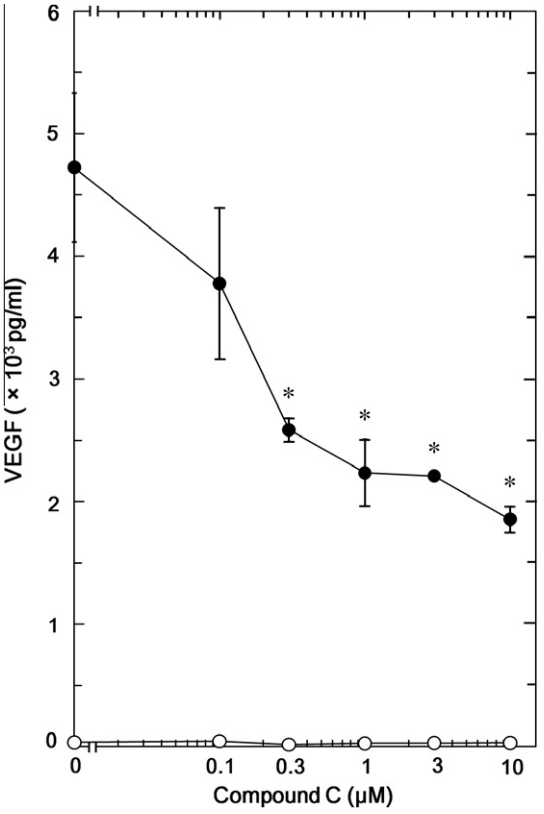

Fig. 2. Effect of AMPK inhibitor on the FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of compound C for 60 min, and then stimulated by 30 ng/ml FGF-2 (●) or vehicle (○) for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of FGF-2 alone.

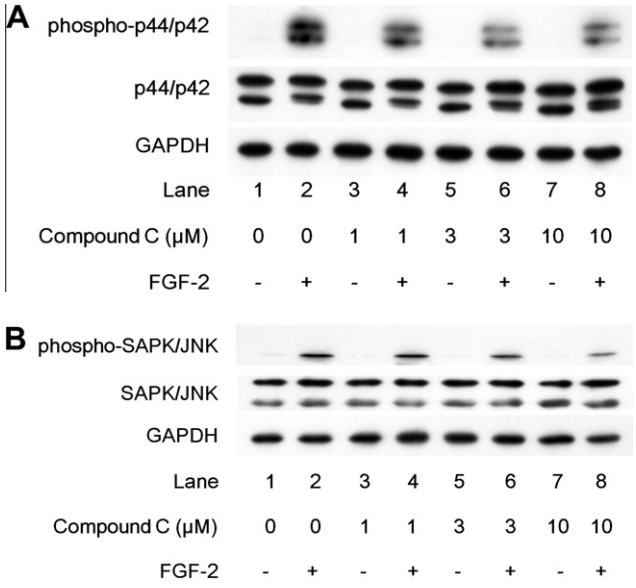

Fig. 3. Effects of AMPK inhibitor on the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of compound C for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against (A) phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase or GAPDH, (B) phospho-specific SAPK/JNK, SAPK/JNK or GAPDH.

suppression of FGF-2-stimulated VEGF synthesis by AMPK inhibitor is mediated through transcriptional events, we next examined the effect of compound C on the FGF-2-induced VEGF mRNA expression by real-time PCR. The FGF-2-induced level of VEGF mRNA was significantly suppressed by compound C, which alone had little effect on the basal level (Fig. 4).

4. Discussion

In the present study, we demonstrated that FGF-2 induced the phosphorylation of AMPK in osteoblast-like MC3T3-E1 cells, using phospho-specific AMPK α-subunit (Thr-172) antibodies. On the other hand, FGF-2 failed to induce the phosphorylation of AMPK α-subunit (Ser-485). It seems unlikely that the phosphorylation of Ser-485 in α-subunit is involved in FGF-2-induced AMPK activation in these cells. In addition, we found that AMPK β-subunit (Ser-108, and Ser-182) was phosphorylated even though without stimulation, and that FGF-2 hardly affected the phosphorylation levels of AMPK β-subunit. It is generally recognized that the phosphorylation of Thr-172 in α-subunit is required for the AMPK activity [5,6]. In addition, it has been shown that phosphorylation at Ser-108 of the β-subunit is recognized to be required for the activation of AMPK, while phosphorylation of Ser-182 affects AMPK localization [28]. Based on our findings, it is most likely that FGF-2 positively regulates AMPK activity via the phosphorylation of AMPK α-subunit (Thr-172) in osteoblast-like MC3T3-E1 cells.

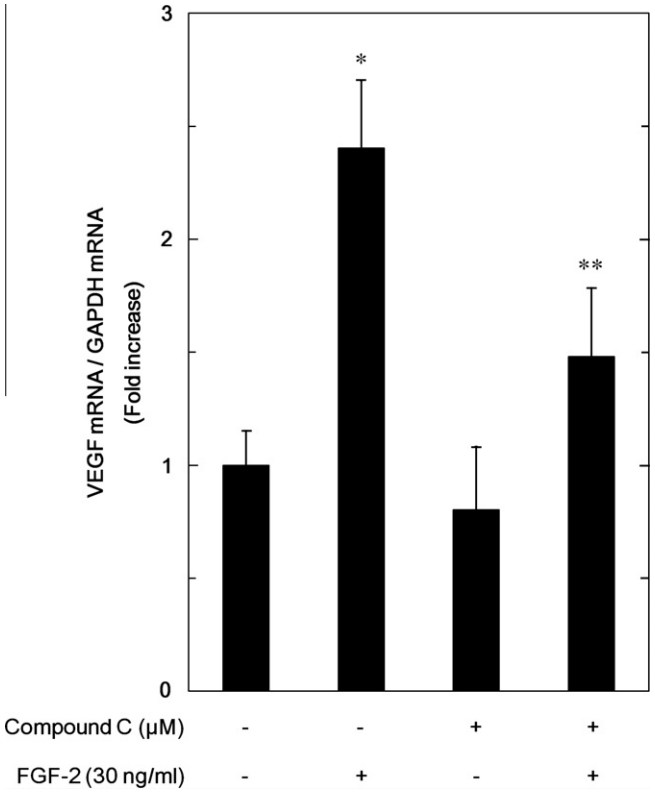

Fig. 4. Effect of AMPK inhibitor on the FGF-2-induced VEGF mRNA expression in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM compound C for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 12 h. Total RNA was isolated and transcribed into cDNA. The expression of VEGF mRNA and GAPDH mRNA were quantified by real-time RT-PCR. VEGF mRNA levels were normalized with those of GAPDH mRNA. Each value represents the mean ± S.E.M. of independent triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of control. $^{*}p < 0.05$, compared to the value of FGF-2 alone.

We next investigated whether AMPK is involved in the FGF-2-stimulated VEGF release or not in osteoblast-like MC3T3-E1 cells. We showed that the FGF-2-stimulated VEGF release was significantly suppressed by compound C, an AMPK inhibitor. Additionally, compound C markedly reduced the VEGF mRNA expression induced by FGF-2. We confirmed that the AMPK inhibitor truly reduced the FGF-2-induced phosphorylation levels of AMPK α-subunit (Thr-172). Therefore, it is probable that activated AMPK positively regulates VEGF synthesis by FGF-2 in osteoblast-like MC3T3-E1 cells. As for the regulatory mechanism underlying FGF-2-stimulated VEGF synthesis, we have previously reported that the FGF-2-stimulated VEGF release is positively regulated by the activation of p44/p42 MAP kinase and SAPK/JNK among the MAP kinase superfamily in osteoblast-like MC3T3-E1 cells [19,20]. In the present study, compound C remarkably reduced the FGF-2-induced phosphorylation levels of both p44/p42 MAP kinase and SAPK/JNK. With regard to the time-dependent effect of FGF-2, we showed that the maximum effect of FGF-2 on the phosphorylation of AMPK α-subunit (Thr-172) was observed at 5 min after the stimulation. In our previous studies [19,20], we have demonstrated that the phosphorylation of p44/p42 MAP kinase or SAPK/JNK reaches the peak at 90 or 20 min, respectively, after the stimulation of FGF-2 in MC3T3-E1 cells. The time course of FGF-2-induced phosphorylation of AMPK appears to be faster than that of p44/p42 MAP kinase or SAPK/JNK. It is reasonable that the activation of p44/p42 MAP kinase and SAPK/JNK subsequently occurs via the activation of AMPK elicited by FGF-2. Therefore, these results suggest that AMPK functions in FGF-2-stimulated VEGF synthesis at a point upstream of p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells. Taking our findings into account as a whole, it is most likely that FGF-2 activates AMPK, resulting in the positive regulation of VEGF synthesis via p44/p42 MAP kinase and SAPK/JNK in osteoblasts.

AMPK is generally recognized to play a pivotal role in the control of metabolic homeostasis, for example, fatty acid synthesis and glycogen synthesis [3]. In bone metabolism, it has been reported that AMPK acts as a positive regulator of bone formation, osteoblast differentiation and mineralization [10,11]. The activation of AMPK reportedly inhibits palmitate-induced apoptosis in osteoblasts [29]. In addition, AMPK is also involved in autophagy in mineralizing tissues [30]. Our present results in bone metabolism could support these reports that AMPK acts as a regulator of bone formation and mineralization. Osteoporosis has recently been a major clinical problem in advanced countries. The pathology of osteoporosis is reduction of bone mineral density, which is a risk of bone fracture [31]. It is well known that FGF-2 expression in osteoblasts is detected during fracture repairing, and that VEGF induces microvasculature in bone tissue [14,17]. Taking these findings into account, it is possible that AMPK provides a new therapeutic aspect for osteoporosis or fracture repairing as well as diabetes and obesity. Further investigations would be required to clarify the details of AMPK in bone metabolism.

In conclusion, our results strongly suggest that AMPK positively regulates FGF-2-stimulated VEGF synthesis through the activation of p44/p42 MAP kinase and SAPK/JNK in osteoblasts.

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