Involvement of AMP-activated protein kinase in thrombin-stimulated interleukin 6 synthesis in osteoblasts

H Tokuda1,2, K Kato2,3, H Natsume2,3, A Kondo2,3, G Kuroyanagi2,3, R Matsushima-Nishiwaki2, Y Ito2, T Otsuka3 and O Kozawa2

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

(Correspondence should be addressed to H Tokuda at Department of Clinical Laboratory, National Center for Geriatrics and Gerontology; Email: tokuda@ncgg.go.jp)

Abstract

We previously demonstrated that thrombin stimulates synthesis of interleukin 6 (IL6), a potent bone resorptive agent, in part via p44/p42 MAP kinase and p38 MAP kinase but not through stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) among the MAP kinase superfamily in osteoblast-like MC3T3-E1 cells. In this study, we investigated the involvement of AMP-activated protein kinase (AMPK), a regulator of energy metabolism, in thrombin-stimulated IL6 synthesis in MC3T3-E1 cells. The phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK, or AMPK was determined by western blot analysis. The release of IL6 was determined by the measurement of IL6 concentration in the conditioned medium using an ELISA kit. The expression of IL6 mRNA was determined by RT-PCR. Thrombin time dependently induced the phosphorylation of AMPK α-subunit (Thr-172). Compound C, an inhibitor of AMPK, dose-dependently suppressed the thrombin-stimulated IL6 release in the range between 0.3 and 10 μM. Compound C reduced thrombin-induced acetyl-CoA carboxylase phosphorylation. The IL6 mRNA expression induced by thrombin was markedly reduced by compound C. Downregulation of AMPK by siRNA suppressed the thrombin-stimulated IL6 release. The thrombin-induced phosphorylation of p44/p42 MAP kinase and p38 MAP kinase was inhibited by compound C, which failed to affect SAPK/JNK phosphorylation. These results strongly suggest that AMPK regulates thrombin-stimulated IL6 synthesis via p44/p42 MAP kinase and p38 MAP kinase in osteoblasts.

Introduction

Interleukin 6 (IL6) is a pleiotropic and proinflammatory cytokine that has crucial effects on a wide range of cell functions such as promoting B-cell differentiation, T-cell activation, and inducing acute-phase proteins (Akira et al. 1993, Heymann & Rousselle 2000, Kwan Tat et al. 2004). It is well recognized that bone metabolism is mainly regulated by two functional cells: osteoblasts and osteoclasts (Karsenty & Wagner 2002). The former cells are responsible for bone formation and the latter cells for bone resorption. These functional cells are closely coordinated via humoral factors or by direct cell-to-cell interaction (Karsenty & Wagner 2002, Kwan Tat et al. 2004). With regard to bone metabolism, IL6 has been shown to stimulate bone resorption and promote osteoclast formation (Ishimi et al. 1990, Roodman 1992, Heymann & Rousselle 2000, Kwan Tat et al. 2004). It has been reported that potent bone resorptive agents such as tumor necrosis factor α, IL1, and prostaglandin estradiol (E2) stimulate IL6 synthesis in osteoblasts (Ishimi et al. 1990, Littlewood et al. 1991, Helle et al. 1998). Currently, accumulating evidence suggests that IL6, which is synthesized and secreted from osteoblasts, plays an important role as a downstream effector of bone resorptive agents in bone metabolism.

AMP-activated protein kinase (AMPK) is generally known to regulate multiple metabolic pathways (Fogarty & Hardie et al. 2010). AMPK has been identified as a mammalian protein kinase that is allosterically activated by AMP and is able to phosphorylate and inactivate enzymes of lipid synthesis (Fogarty & Hardie et al. 2010). It is currently recognized that AMPK is a key sensing enzyme in the regulation of cellular energy homeostasis (Hardie et al. 2006, Lage et al. 2008, Steinberg & Kemp 2009). AMPK is activated in mammalian cells by a variety of physiological and pathological stresses that increase the intracellular AMP:ATP ratio, either by increasing ATP consumption or by decreasing ATP production. AMPK acts to restore cellular energy balance by ATP-generating pathways.
such as fatty acid oxidation while simultaneously inhibiting ATP using pathways. Regarding AMPK in bone metabolism, it has recently been reported that AMPK regulates bone formation and bone mass in vitro (Shah et al. 2010). We have previously shown that AMPK plays a role in vascular endothelial growth factor synthesis in osteoblasts (Kato et al. 2010). However, the exact role of AMPK in bone metabolism has not yet been elucidated.

It is well recognized that thrombin, a serine protease, plays a crucial role in the blood coagulation cascade through its cleavage of fibrinogen to fibrin (Lane et al. 2005). Evidence is accumulating that thrombin has receptor-mediated effects on a variety of cells including osteoblasts via protease-activated receptors (Mackie et al. 2008). In bone metabolism, it has been shown that thrombin stimulates bone resorption (Mackie et al. 2008). As for osteoblasts, thrombin reportedly stimulates their proliferation and IL6 secretion and suppresses alkaline phosphatase activity, a marker of osteoblast differentiation (Mackie et al. 2008). We have previously demonstrated that thrombin induces the activation of protein kinase C via phosphatidylcholine-hydrolyzing phospholipase D but not phosphoinositide-hydrolyzing phospholipase C in osteoblast-like MC3T3-E1 cells and that its proliferative effect depends on phospholipase D activation (Suzuki et al. 1996). Regarding IL6 synthesis in osteoblasts, we have reported that thrombin stimulates IL6 synthesis in these cells and that p44/p42 MAP kinase and p38 MAP kinase among the MAP kinase superfamily play a role in thrombin-induced IL6 synthesis (Kozawa et al. 1997, Kato et al. 2011). However, the detailed mechanism behind thrombin-stimulated IL6 synthesis in osteoblasts remains to be clarified.

In this study, we investigated the involvement of AMPK in thrombin-stimulated IL6 synthesis in osteoblast-like MC3T3-E1 cells. We here show that AMPK positively regulates thrombin-stimulated IL6 synthesis at a point upstream from p44/p42 MAP kinase and p38 MAP kinase activation in these cells.

Materials and methods

Materials

Thrombin, mouse IL6 ELISA kit, and bone morphogenetic protein 2 (BMP2) ELISA kit were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Compound C was obtained from Calbiochem–Novabiochem Co. (La Jolla, CA, USA). Phospho-specific AMPK α-subunit (Thr-172) antibodies, phospho-specific AMPK α-subunit (Ser-485) antibodies, AMPK α-subunit antibodies, phospho-specific AMPK β-subunit (Ser-108) antibodies, phospho-specific AMPK β-subunit (Ser-182) antibodies, AMPK β-subunit antibodies, phospho-specific acetyl-CoA carboxylase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) antibodies, and SAPK/JNK antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). GAPDH antibodies were obtained from Santa Cruz Biotechnology, Inc. An ECL western blotting detection system was purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). Control siRNA (Silencer Negative Control no. 1 siRNA) and AMPKα1 siRNA (Flexi Tube siRNA, Mm_Prrka1) were purchased from Ambion (Austin, TX, USA) and Qiagen respectively. siLentFect was purchased from Bio-Rad. Trizol reagent and omniscript reverse transcriptase kit were purchased from Invitrogen and Qiagen respectively. Fast-start DNA Master SYBR Green I was purchased from Roche Diagnostics. Other materials and chemicals were obtained from commercial sources.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells that have been derived from newborn mouse calvaria (Sudo et al. 1983) were maintained as described previously (Kozawa et al. 1992). 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% CO2/95% air. The cells were seeded onto 35 mm (5×104) or 90 mm (2×105) diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

IL6 assay

The cultured cells were stimulated by 1 U/ml thrombin in 1 ml 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 concentration of IL6 was measured by IL6 ELISA kit.

BMP2 assay

The cultured cells were pretreated with 10 μM compound C or vehicle for 60 min and then stimulated by 1 U/ml thrombin or vehicle for 48 h. The conditioned medium was collected at the end of the incubation, and the concentration of BMP2 was measured by BMP2 ELISA kit.
siRNA transfection

To knockdown AMPK in MC3T3-E1 cells, the cells were transfected with negative control siRNA or AMPK siRNA using siLentFect according to the manufacturer’s protocol. In brief, the cells (1 × 10^5 cells) were seeded onto 35 mm diameter dishes in α-MEM containing 10% FCS and subcultured for 48 h. The cells were then incubated at 37°C with 50 nM siRNA–siLentFect complexes. After 24 h, the medium was exchanged to α-MEM containing 0.3% FCS. They were then stimulated by 1 U/ml thrombin in α-MEM containing 0.3% FCS for 48 h.

Western blot analysis

A western blot analysis was performed as described previously (Kato et al. 1996). The cultured cells were stimulated by thrombin or vehicle 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 PBS and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris–HCl, pH 6.8, 3% SDS, 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at ×125 000 g for 10 min at 4°C. SDS–PAGE was performed according to Laemmli (1970) using 10% polyacrylamide gel. The protein (20 μg) was fractionated and transferred onto an Immun-Blot PVDF membrane (Bio-Rad). Membranes were blocked with 5% fat-free dry milk in Tris-buffered saline–Tween (TBS–T; 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 2 h before incubation with the primary antibodies. Phospho-specific AMPK α-subunit (Thr-172) antibodies, phospho-specific AMPK α-subunit (Ser-182) antibodies, AMPK α-subunit antibodies, phospho-specific AMPK β-subunit (Ser-108) antibodies, phospho-specific AMPK β-subunit (Ser-182) antibodies, AMPK β-subunit antibodies, phospho-specific acetyl-CoA carboxylase antibodies, GAPDH antibodies, phospho-specific p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, or SAPK/JNK antibodies were used as primary antibodies. Peroxidase-labeled antibodies raised in goat against rabbit IgG (KPL, Inc., Gaithersburg, MD, USA) were used as secondary antibodies. The primary and secondary antibodies were diluted at 1:1000 with 5% fat-free dry milk in TBS–T. Peroxidase activity on the membrane was visualized on an X-ray film by means of the ECL western blotting detection system.

Real-time RT-PCR

The cultured cells were stimulated with 1 U/ml thrombin for the indicated periods. 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 the capillaries and Fast-Start DNA Master SYBR Green I provided with the kit. Sense and antisense primers for mouse IL6 or GAPDH mRNA were purchased from Takara Bio, Inc. (Tokyo, Japan; primer set ID: MA039013). The amplified products were determined by a melting curve analysis and agarose electrophoresis. IL6 mRNA levels were normalized with those of GAPDH mRNA.

Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The quantification of the data of western blot was performed using scanner and image analysis software (ImageJ version 1.44; National Institutes for Health, USA).

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 ± S.E.M. of triplicate independent determinations.

Results

Effect of thrombin on the phosphorylation of AMPK in MC3T3-E1 cells

It has been reported that the phosphorylation of AMPK is essential for its activation (Hawley et al. 1996). Therefore, we first examined the effect of thrombin on the phosphorylation of AMPK in osteoblast-like MC3T3-E1 cells in order to investigate whether thrombin could elicit the activation of AMPK in osteoblasts. Thrombin remarkably induced the phosphorylation of AMPK α-subunit (Thr-172; Fig. 1A). On the other hand, thrombin did not induce the phosphorylation of AMPK α-subunit (Ser-485). In addition, the levels of phosphorylated AMPK β-subunit (Ser-108) or phosphorylated AMPK β-subunit (Ser-182) were not affected by thrombin (Fig. 1A). The effect of thrombin on the phosphorylation of AMPK α-subunit (Thr-172) reached its peak within 2 min and thereafter decreased.
Effect of compound C on thrombin-stimulated IL6 release in MC3T3-E1 cells

We previously showed that thrombin stimulates IL6 synthesis in osteoblast-like MC3T3-E1 cells (Kozawa et al. 1997). In order to clarify whether AMPK plays a role in thrombin-induced synthesis of IL6 in these cells, we next examined the effect of compound C, an inhibitor of AMPK (Zhou et al. 2001) on thrombin-stimulated release of IL6. Compound C, which by itself had little effect on the basal levels of IL6, significantly reduced thrombin-stimulated IL6 release (Fig. 1B). The suppressive effect of compound C on the IL6 release was dose-dependent in the range between 0.3 and 10 μM. Compound C (10 μM) caused ~60% inhibition in the thrombin effect.

Effect of compound C on BMP2 release with or without thrombin in MC3T3-E1 cells

It has recently been reported that compound C, also known as dorsomorphin, has AMPK-independent actions and inhibits the signaling of BMP (Yu et al. 2008). To clarify whether BMP is involved in the inhibitory effect of compound C, we examined the effect of thrombin on BMP release and the effect of compound C on thrombin-induced BMP release in the osteoblast-like MC3T3-E1 cells. We found that the basal level of BMP2 was below 62.5 pg/ml for control, <62.5 pg/ml for 1 U/ml thrombin alone, <62.5 pg/ml for 10 μM compound C alone, and <62.5 pg/ml for 1 U/ml thrombin with 10 μM compound C pretreatment). Therefore, it seems unlikely that BMP is involved in the inhibitory effect of compound C on IL6 synthesis induced by thrombin.

Effects of thrombin on the phosphorylation of acetyl coenzyme A carboxylase in MC3T3-E1 cells

It is generally recognized that acetyl coenzyme A (acyl-CoA) carboxylase, which plays a central role in the regulation of lipid synthesis, is a direct substrate of AMPK (Fogarty & Hardie 2010). Thrombin markedly stimulated the phosphorylation of acetyl-CoA carboxylase (Fig. 2). The effect of thrombin on the acetyl-CoA carboxylase phosphorylation reached its peak within 2 min and thereafter decreased. We found that compound C truly suppressed the thrombin-induced phosphorylation of acetyl-CoA carboxylase (Fig. 3).

Effect of compound C on thrombin-stimulated IL6 mRNA expression in MC3T3-E1 cells

In order to elucidate whether the suppression of thrombin-stimulated IL6 synthesis by compound C is
mediated via transcriptional event, we examined the effect of compound C on the thrombin-induced expression level of IL6 mRNA. Compound C, which by itself had little effect on the basal expression level, almost completely reduced the thrombin-induced level of IL6 mRNA (Fig. 4).

Effect of AMPK-knockdown on thrombin-stimulated IL6 release in MC3T3-E1 cells

We examined the effect of AMPK downregulation by siRNA on thrombin-induced IL6 release in osteoblast-like MC3T3-E1 cells. Thrombin-stimulated IL6 release was significantly diminished in AMPK-knockdown cells in comparison with control cells (Table 1). Treatment with AMPK siRNA caused almost complete reduction in the thrombin effect.

Effects of compound C on the thrombin-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells

It is currently known that three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mammalian cells to transduce the various messages of a variety of agonists (Kyriakis & Avruch 2001). In our previous study (Kato et al. 2011), we have shown that thrombin activates p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK in osteoblast-like MC3T3-E1 cells, and p44/p42 MAP kinase and p38 MAP kinase, but not SAPK/JNK, act as positive regulators in the thrombin-stimulated IL6 synthesis. In order to investigate whether the effect of AMPK on thrombin-stimulated IL6 synthesis is due to the activation of these MAP kinases in MC3T3-E1 cells, we next examined the effects of compound C on the thrombin-induced phosphorylation of p44/p42 MAP kinase or p38 MAP kinase. Compound C remarkably reduced the phosphorylation of p44/p42 MAP kinase (Fig. 5) and p38 MAP kinase (Fig. 6) induced by thrombin in the range between 1 and 10 μM. In addition, we found that thrombin-induced phosphorylation of SAPK/JNK was not suppressed by 10 μM of compound C (Fig. 7).

Discussion

In this study, we demonstrated that thrombin markedly induced the phosphorylation of AMPK α-subunit...
Additionally, we found that compound C, acetyl-CoA carboxylase were closely related in their thrombin on the phosphorylation of both AMPK and Fogarty & Hardie 2010). It seems that the effects of carboxylase causes downregulation of lipid synthesis substrate of AMPK, and the activation of acetyl-CoA. CoA carboxylase phosphorylation in MC3T3-E1 cells. We showed that thrombin remarkably induced acetyl-CoA is essential for enzyme activation (Hawley 1996, Hawley et al. 1996). Among the three subunits of AMPK, the α-subunit is recognized to be a catalytic site, whereas the β- and γ-subunits are regulatory sites. It has been reported that the phosphorylation of AMPK α-subunit at Thr-172 is essential for enzyme activation (Hawley et al. 1996). We showed that thrombin remarkably induced acetyl-CoA carboxylase phosphorylation in MC3T3-E1 cells. Acetyl-CoA carboxylase is well known as a direct substrate of AMPK, and the activation of acetyl-CoA carboxylase causes downregulation of lipid synthesis such as fatty acid and cholesterol (Hawley et al. 1996, Fogarty & Hardie 2010). It seems that the effects of thrombin on the phosphorylation of both AMPK and acetyl-CoA carboxylase were closely related in their time course. Additionally, we found that compound C, an inhibitor of AMPK (Zhou et al. 2001), truly suppressed thrombin-induced phosphorylation levels of acetyl-CoA carboxylase. Taking our findings into account, it is most likely that thrombin stimulates the activation of AMPK in osteoblast-like MC3T3-E1 cells.

We next investigated whether AMPK activation plays a role in thrombin-stimulated IL6 synthesis in osteoblast-like MC3T3-E1 cells. The release of IL6 stimulated by thrombin was significantly reduced by compound C in a dose-dependent manner. These findings suggest that thrombin-activated AMPK is involved in IL6 synthesis in MC3T3-E1 cells. In addition, we demonstrated that compound C inhibited the IL6 mRNA expression induced by thrombin. Moreover, we showed that thrombin-stimulated IL6 release was significantly diminished in AMPK-knockdown cells in comparison with the control cells. Therefore, it is probable that thrombin-induced IL6 synthesis is mediated through the activation of AMPK in osteoblast-like cells. Furthermore, we confirmed that thrombin failed to affect BMP2 release with or without compound C, also known as dorsomorphin, which reportedly inhibits BMP signaling (Yu et al. 2008). It seems unlikely that BMP2 is involved in the effect of compound C on IL6 synthesis induced by thrombin in these cells. Collectively, therefore, our results suggest that thrombin activates AMPK, which functions as a positive regulator in IL6 synthesis in osteoblast-like MC3T3-E1 cells.

In a previous study (Kato et al. 2011), we have reported that thrombin stimulates p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK in osteoblast-like MC3T3-E1 cells and that p44/p42 MAP kinase and p38 MAP kinase among them act as positive regulators in thrombin-stimulated IL6 synthesis, while SAPK/JNK is not involved. Therefore, we investigated the relationship between AMPK and MAP kinase signaling, p44/p42 MAP kinase or p38 MAP kinase in thrombin-stimulated IL6 synthesis. The thrombin-induced phosphorylation levels of p44/p42 MAP kinase or p38 MAP kinase were remarkably attenuated by compound C in a dose-dependent manner. Additionally, we found that compound C failed to affect the thrombin-induced phosphorylation of SAPK/JNK. Based on our findings, it is most likely that AMPK Table 1 Effect of AMPK siRNA on thrombin-induced IL6 release in MC3T3-E1 cells. The cultured cells were transfected with 50 nM control siRNA or 50 nM AMPK siRNA using the siLentFect.

<table>
<thead>
<tr>
<th>AMPK siRNA</th>
<th>Thrombin</th>
<th>IL6 (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>7.8</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>34.5±7.3*</td>
</tr>
<tr>
<td>AMPK</td>
<td>–</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td>AMPK</td>
<td>+</td>
<td>&lt;7.8</td>
</tr>
</tbody>
</table>

*P<0.05 compared with the value of vehicle with control siRNA transfection. †P<0.05 compared with the value of thrombin with control siRNA transfection.

Figure 5 Effect of compound C on the thrombin-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of compound C for 60 min and then stimulated by 1 U/ml thrombin or vehicle for 15 min. The extracts of cells were subjected to SDS–PAGE with subsequent western blot analysis using antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantification of the levels of thrombin-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean±S.E.M. of triplicate determinations. *P<0.05 compared with the value of control. **P<0.05 compared with the value of thrombin alone.
regulates thrombin-stimulated IL6 synthesis via the activation of both p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. In this study, we showed that the maximum effect of thrombin on the phosphorylation of AMPK α-subunit (Thr-172) was observed within 2 min after the stimulation. On the other hand, we previously showed that the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase reached the peak at 5 and 15 min after the stimulation of thrombin respectively (Kato et al. 2011). The time course of thrombin-induced phosphorylation of AMPK appears to be faster than those of p44/p42 MAP kinase and p38 MAP kinase. Therefore, it seems reasonable that thrombin-induced activation of these MAP kinases subsequently occurs after the activation of AMPK in MC3T3-E1 cells. Taking our findings into account as a whole, these results strongly suggest that AMPK acts at a point upstream from p44/p42 MAP kinase and p38 MAP kinase and regulates thrombin-stimulated IL6 synthesis in osteoblast-like MC3T3-E1 cells.

AMPK is well known as a key sensor of changes in cellular energy homeostasis such as lipid synthesis (Rutter & Leclerc 2009). With regard to bone metabolism, it has been shown that AMPK stimulates bone formation and mineralization (Kanazawa et al. 2008, Shah et al. 2010). It has recently been shown that AMPK stimulates osteoblast differentiation via induction of RUNX2 expression (Jang et al. 2011). In addition, the activation of AMPK reportedly inhibits palmitate-induced apoptosis in osteoblasts (Kim et al. 2008). Our present results suggest that AMPK functions as a positive regulator in thrombin-stimulated IL6 synthesis in osteoblasts. It is well known that IL6 produced by osteoblasts is a potent bone resorptive agent and induces osteoclast formation (Ishimi et al. 1990, Kwan Tat et al. 2004). Therefore, our present findings lead us to speculate that thrombin-activated AMPK in osteoblasts plays as a positive regulator of bone resorption via the fine-tuning of the local cytokine network, such as synthesis of IL6. Thus, AMPK would be one of the essential molecules for bone remodeling process. 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 factor for bone fracture (Unnanuntana et al. 2010). The signaling pathway of AMPK in osteoblasts might be a new candidate as a molecular

**Figure 6** Effect of compound C on the thrombin-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of compound C for 60 min and then stimulated by 1 U/ml thrombin or vehicle for 5 min. The extracts of cells were subjected to SDS–PAGE with subsequent western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantification of the levels of thrombin-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean ± S.E.M. of triplicate determinations. *P < 0.05 compared with the value of control. **P < 0.05 compared with the value of thrombin alone.

**Figure 7** Effect of compound C on the thrombin-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM compound C or vehicle for 60 min and then stimulated by 1 U/ml thrombin or vehicle for 15 min. The extracts of cells were subjected to SDS–PAGE with subsequent western blot analysis using antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantification of the levels of thrombin-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean ± S.E.M. of triplicate determinations.
therapeutic target of bone metabolic disease such as osteoporosis. However, the exact role of AMPK in bone metabolism has not yet been fully clarified. Further investigations are necessary to elucidate the details of AMPK in bone metabolism.

In conclusion, our results strongly suggest that AMPK positively regulates thrombin-stimulated IL6 synthesis via p44/p42 MAP kinase and p38 MAP kinase in osteoblasts.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This investigation was supported in part by a grant-in-aid for scientific research (grants no.: 16590873 and 16591482) for the Ministry of Education, Science, Sports and Culture of Japan, the Foundation for Growth Science and the Research Funding for Longevity Sciences (21-4, 22-4, and 23-9) from National Center for Geriatrics and Gerontology (NCGG), Japan.

Acknowledgements

The authors are very grateful to Yoko Kawamura for her skillful technical assistance.

References

Akira S, Taga T & Kishimoto T 1993 Interleukin-6 in biology and medicine. *Advances in Immunology* 54 1–78.


Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D & Hardie DG 1996 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. *Journal of Biological Chemistry* 271 27879–27887. (doi:10.1074/jbc.271.44.27879)


Kanazawa I, Yamaguchi T, Yano S, Yamauchi M & Sugimoto T 2008 Metformin enhances the differentiation and mineralization of osteoblastic MC3T3-E1 cells via AMPK activation as well as eNOS and BMP-2 expression. *Biochemical and Biophysical Research Communications* 375 414–419. (doi:10.1016/j.bbrc.2008.08.034)


Received in final form 23 May 2011
Accepted 28 May 2012
Made available online as an Accepted Preprint 29 May 2012