Activation of AMP kinase and inhibition of Rho kinase induce the mineralization of osteoblastic MC3T3-E1 cells through endothelial NOS and BMP-2 expression

Ippei Kanazawa, Toru Yamaguchi, Shozo Yano, Mika Yamauchi, and Toshitsugu Sugimoto

Department of Internal Medicine 1, Shimane University Faculty of Medicine, Enya-cho, Japan

Submitted 6 August 2008; accepted in final form 6 November 2008

AMP-ACTIVATED PROTEIN KINASE (AMPK) is found in single-cell eukaryotes such as a yeast Saccharomyces cerevisiae and a primitive protist Giardia lamblia (5, 14) and is considered as an evolutionarily conserved sensor of cellular energy status. Recent data have demonstrated that the enzyme also plays a critical role in systemic energy balance. AMPK integrates nutritional and hormonal signals in peripheral tissues and the hypothalamus (17). For example, adipose tissue acts as an endocrine organ that secretes a large number of hormones and cytokines that have systemic effects on processes such as glucose and lipid homeostasis, body weight regulation, blood pressure, and immune function (21). These molecules have been termed adipokines, the representatives of which are adiponectin and leptin, and many of their metabolic actions are mediated by AMPK.

AMPK are known to be expressed ubiquitously, including bone (8, 13), and could possibly influence bone metabolism. Recently, we have demonstrated for the first time that adiponectin and 5-aminimidazole-4-carboxamide-β-d-ribo-nucleoside (AICAR), both of which are able to activate AMPK (6, 11), stimulated the differentiation and mineralization of osteoblastic MC3T3-E1 cells (18) and suggested that AMPK may have an important function in bone tissue as well as adipose tissue.

Activation of AMPK suppresses 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (3, 12), which acts as a rate-limiting enzyme for endogenous cholesterol synthesis and is considered as a key enzyme in the mevalonate pathway. Its inhibitors (statins) are widely used as cholesterol-lowering medicines for the prevention of coronary heart disease. Moreover, statins have recently been shown to exert pleiotropic effects on various cells, which may not be directly related to cholesterol synthesis. Accumulating evidence has shown that statins also prevent the synthesis of isoprenoid intermediates such as farnesyl pyrophosphate and geranyl-geranyl pyrophosphate (GGPP) (12) as well as small GTP-binding proteins (G proteins) such as Rho, Ras, and Rab, all of which are located in the downstream of HMG-CoA reductase and have recently attracted widespread attention as the most important factors of pleiotropic effects by statins. In particular, the small GTPase Rho and one of its downstream effectors, the Rho-associated protein kinase (ROK), have been intensely investigated about pleiotropic effects on various cells, which may not be directly related to cholesterol synthesis. Indeed, fasudil hydrochloride, a specific inhibitor of ROK, attracted widespread attention as the most important factors of pleiotropic effects by statins. In particular, the small GTPase Rho and one of its downstream effectors, the Rho-associated protein kinase (ROK), have been intensely investigated about pleiotropic effects on various cells, which may not be directly related to cholesterol synthesis. Indeed, fasudil hydrochloride, a specific inhibitor of ROK, attracted widespread attention as the most important factors of pleiotropic effects by statins. In particular, the small GTPase Rho and one of its downstream effectors, the Rho-associated protein kinase (ROK), have been intensely investigated about pleiotropic effects on various cells, which may not be directly related to cholesterol synthesis.

Address for reprint requests and other correspondence: Toru Yamaguchi, Dept. of Internal Medicine 1, Shimane Univ. Faculty of Medicine, 89-1 Enya-cho, Izumo 693-8501, Japan (e-mail: yamaguch@med.shimane-u.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpendo.org
0193-1849/09 $8.00 Copyright © 2009 the American Physiological Society
Statins are also known to affect bone, by inducing bone morphogenetic protein-2 (BMP-2) and eNOS expression in osteoblasts and stimulating bone formation (10, 30). Moreover, inhibition of ROK by fasudil has been shown to increase BMP-2 mRNA expression in human osteoblasts (31). Thus agents activating AMPK as well as inhibiting either HMG-CoA reductase or ROK could modulate the mevalonate pathway and are expected to be candidate drugs not only curing the cardiovascular disease but also promoting bone formation for the treatment of osteoporosis.

The present study was undertaken to investigate the mechanisms by which AMPK activation by AICAR as well as ROK inhibition by fasudil control the differentiation of osteoblastic MC3T3-E1 cells. The results showed that these agents promoted the mineralization of the cells through enhancing eNOS and BMP-2 expression, confirming the importance of the mevalonate pathway for osteoblast function.

MATERIALS AND METHODS

Materials. Cell culture medium and supplements were purchased from GIBCO-BRL (Rockville, MD). AICAR was purchased from Sigma-Aldrich (St. Louis, MO), and fasudil was a kind gift from Asahi-kasei (Tokyo, Japan). Mevalonate and GGPP were purchased from Sigma (St. Louis, MO). PD-98059, a specific extracellular signal-regulated kinase (ERK) inhibitor, was purchased from Wako Chemical (Osaka, Japan). A5-nitro-l-arginine methyl ester hydrochloride (l-NNAME), a nitric oxide synthase (NOS) inhibitor, and noggin, a BMP antagonist, were purchased from To errs (El lisville, MO) and PeproTech (Rocky Hill, NJ), respectively. For Western blot analysis, total and phospho-Akt antibodies were purchased from R & D systems (Minneapolis, MN) and Rockland (Gilbertsville, PA), respectively, and total and phospho-ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA). For ROK activity analysis, a ROK assay enzyme-linked immunosorbent assay (ELISA) kit was purchased from Cyclex (Nagano, Japan). All other chemicals were of the highest grade available commercially.

Cell cultures. MC3T3-E1 cells, a clonal osteoblastic cell line isolated from calvariae of late-stage mouse embryo (36), were kindly provided by Dr. H. Kodama (Ohu Dental College). MC3T3-E1 cells were cultured in α-minimum essential medium (α-MEM). This medium was supplemented with 10% FBS and 1% penicillin-streptomycin (GIBCO-BRL) in 5% CO2 at 37°C. The medium was changed two times a week. For mineralization assay, MC3T3-E1 cells were cultured in α-MEM containing 10% FBS, 1% penicillin-streptomycin, and 10 mM β-glycerophosphate for 2 wk after reaching confluence.

Real-time PCR quantification of gene expression. SYBR green was used to perform quantitative determinations of the mRNAs for BMP-2, eNOS, osteocalcin (OCN), and a housekeeping gene, 36B4, according to an optimized protocol (27, 41). Total RNA was taken from cultured MC3T3-E1 cells using Trizol reagent (Invitrogen, San Diego, CA) according to the manufacturer’s recommended protocol. Total RNA (2 μg) was employed for the synthesis of single-stranded cDNA (cDNA synthesis kit; Invitrogen). The sense and antisense primers were designed using the Primer Express version 2.0.0 (Applied Biosystems) based on published cDNA sequences. Primer sequences were as follows: mouse BMP-2, 5′-GCTTCACAGATAAGGCAATGTCG-3′ and 5′-GCTTCACGTGTTTGGTGTTTG-3′; mouse eNOS, 5′-CCTTCCGTACAGCCAGCA-3′ and 5′-CAGGATCCTTTACGATGGCTGA-3′; mouse OCN, 5′-GTGGTTGACGAGCTATACG-3′ and 5′-GAGGACAGGGGAGATCAGT-3′; and mouse 36B4, 5′-AAGGGCTCTTGGTTGCTGTTT-3′ and 5′-GGGGAGGGGGAGGGGGAGGGG-3′. Real-time PCR was performed using 1 μl of cDNA in a 25-μl reaction volume with ABI PRISM 7000 (PE Applied Biosystems). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the QuantiTech SYBR PCR kit (Qiagen, Valencia, CA) to allow for quantitative detection of the PCR product. The temperature profile of the reaction was 95°C for 15 min, 40 cycles of denaturation at 94°C for 15 s, and annealing and extension at 60°C for 1 min. 36B4 was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription.

Alkaline phosphatase staining. Cultured cells were rinsed in PBS, fixed with 1% formaldehyde, rinsed again with PBS, and then overlaid with 1.0 ml of 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate plus 0.3 mg/ml nitro blue tetrazolium chloride in 0.1 M Tris-HCl pH 9.5, 0.01 N NaOH, and 0.05 M MgCl2, followed by incubation at room temperature for 6 h in the dark.

Assay of alkaline phosphatase activity. After reaching confluence, cells in 24-well plates were rinsed three times with PBS, and 600 μl of distilled water were added to each well and sonicated. The protein assay was performed with the bicinchoninic acid protein assay reagent.
Alkaline phosphatase (ALP) activity was assayed by a method modified from that of Lowry et al. (28). In brief, the assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol, 1 mM MgCl₂, 8 mM p-nitrophenyl phosphate disodium, and cell homogenates. After 4 min of incubation at 37°C, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol. Each value was normalized to the protein concentration.

Mineralization of MC3T3-E1 cells was determined in 6-well or 12-well plates using von Kossa staining or Alizarin red staining. The cells were stained with 2% AgNO₃ and fixed with 2.5% NaS₂O₃ by the von Kossa method to detect phosphate deposits in bone nodules (40). At the same time, the order plates were fixed with ice-cold 70% ethanol and stained with Alizarin red to detect calcification. For quantification, cells stained with Alizarin red (n/H₁₁₀²₂₂ 5/H₁₁₀²₅₆) were destained with ethlypyridium chloride, then the extracted stain was transferred to a 96-well plate, and the absorbance at 550 nm was measured using a microplate reader, as previously described (35).

ROK activity assay. Cells were rinsed with ice-cold PBS and scraped on ice in a lysis buffer (Cell Signaling Technology) that contained 20 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM EGTA, 1 mM Na₂EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO₄, and 1 μg/ml leupeptin. The cell lysates were then sonicated for 30 s. After the cell lysates were centrifuged at 15,000 g for 10 min, supernatants were collected for assessing ROK activities using the ROK assay kit (Cyclex) as indicated by the manufacturer. Briefly, 10 μl of the supernatants of lysed cells were added in 96-well plates precoated with a substrate corresponding to the COOH terminus of the recombinant myosin-binding subunit of myosin phosphate (MSB), which contains a threonine residue that may be phosphorylated by ROK. Subsequently, 90 μl of a kinase reaction buffer (containing 0.1 mM ATP) were added, incubated for 30 min at room temperature, washed five times in a washing buffer provided by the kit, and incubated with 100 μl of a horseradish peroxidase-conjugated monoclonal antiphospho-specific MSB antibody, which specifically detects the phosphorylated form of threonine 697 on MSB (provided by the kit). The colored products were developed by incubating with 100 μl of a horseradish peroxidase substrate tetramethylbenzidine at room temperature for 10 min. The reaction was stopped by adding 100 μl of stop solution containing 0.5 M H₂SO₄, and the absorbance was read at 450 nm. Each value was normalized to the protein concentration.

Statistics. Results are expressed as means ± SE. Statistical evaluations for differences between groups were carried out using one-way ANOVA followed by Fisher’s protected least significant difference. For all statistical tests, a value of P < 0.05 was considered to be a statistically significant difference.

Fig. 2. Effect of AICAR on bone morphogenetic protein (BMP)-2 mRNA expression in osteoblastic MC3T3-E1 cells via the mevalonate pathway. A: BMP-2 mRNA expression was significantly increased by AICAR (0.5 mM) after 7, 14, and 21 experiment days in MC3T3-E1 cells. B: AICAR stimulated BMP-2 mRNA expression at 0.1 and 0.5 mM after 14 days. C: AICAR (0.5 mM)-augmented BMP-2 mRNA expression was reversed by the addition of 1 mM mevalonate or 5 μM GGPP, the downstream metabolites of 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase, after 7, 14, and 21 days, suggesting that AICAR could stimulate BMP-2 mRNA expression via suppressing HMG-CoA reductase in MC3T3-E1 cells. Results are expressed as the mean ± SE fold increase over control values (n = 7). *P < 0.05.

Fig. 3. Effect of AICAR on endothelial nitric oxide synthase (eNOS) mRNA expression in osteoblastic MC3T3-E1 cells via the mevalonate pathway. AICAR (0.5 mM) stimulated eNOS mRNA expression after 7 days (A). AICAR (0.5 mM)-augmented eNOS mRNA expression was reversed by the addition of 1 mM mevalonate or 5 μM GGPP, the downstream metabolites of HMG-CoA reductase, after 7 days (B), suggesting that AICAR could stimulate eNOS mRNA expression via suppressing HMG-CoA reductase in MC3T3-E1 cells. Results are expressed as the mean ± SE fold increase over control values (n = 9). *P < 0.05.
RESULTS

Effect of AICAR on the differentiation and mineralization of MC3T3-E1 cells via the mevalonate pathway. We first investigated whether or not AICAR, an AMPK activator, could augment the mineralization of MC3T3-E1 cells through modulating the mevalonate pathway. Mineralization of MC3T3-E1 cells was augmented by 0.1 mM AICAR by both von Kossa and Alizarin red stainings for 21 days (Fig. 1, A and B). AICAR (0.5 mM) significantly increased OCN mRNA expression levels by real-time PCR for 14 days (*P < 0.001) (Fig. 1C) and mineralization (*P < 0.001) (Fig. 1, A and B), although mevalonate or GGPP did not affect the expression of OCN mRNA or the mineralization of the cells in the absence of AICAR (data not shown).

Effect of AICAR on BMP-2 mRNA expression in osteoblastic MC3T3-E1 cells via the mevalonate pathway. Next, we examined whether or not activation of AMPK by AICAR and subsequent inhibition of HMG-CoA reductase could stimulate BMP-2 mRNA expression in osteoblastic MC3T3-E1 cells. AICAR was added after the cells reached confluency on 5 days, and total RNA was collected on 7, 14, and 21 days. AICAR (0.5 mM) significantly increased the BMP-2 mRNA expression levels in a time-dependent manner until 21 days by 10.220.33.3 on June 27, 2017 http://ajpendo.physiology.org/ Downloaded from

Fig. 4. Effects of a nitric oxide synthase (NOS) inhibitor or a BMP antagonist on the AICAR-induced mineralization as well as OCN mRNA expression in osteoblastic MC3T3-E1 cells. Coincubation with 1 mM N\(^\text{\textsuperscript{G}}\) - nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor, or 100 ng/ml noggin, a BMP antagonist, significantly reversed the AICAR-induced enhancement of mineralization by both von Kossa and Alizarin red stainings and alizarin red quantification after 21 days (*P < 0.05) (A and B, respectively) and that of OCN mRNA expression by real-time PCR after 7 days (*P < 0.05) (C) in MC3T3-E1 cells. Results from real-time PCR were expressed as the mean ± SE fold increase over control values (n = 10). *P < 0.05.

Fig. 5. Effects of AICAR on the protein kinase B (Akt) and extracellular signal-regulated kinase (ERK) activation as well as BMP-2 and eNOS mRNA expression in osteoblastic MC3T3-E1 cells via the ERK pathway. AICAR (0.5 mM) was able to phosphorylate Akt (A) and ERK (B) in MC3T3-E1 cells in a time-dependent manner for up to 60 min. The result was representative of three different experiments. Coincubation with 25 \(\mu\)M PD-98059 significantly reversed the AICAR (0.5 mM)-induced enhancement of BMP-2 mRNA expression at 48 h (C) and eNOS mRNA expression at 24 and 48 h (D). Results are expressed as the mean ± SE fold increase over control values (n = 9). *P < 0.05.
real-time PCR ($P < 0.05$) (Fig. 2A). AICAR also significantly increased BMP-2 mRNA expression levels at 0.1 and 0.5 mM after 14 days ($P < 0.05$) (Fig. 2B). Next, experiments were performed to clarify whether or not these effects would be via the mevalonate pathway. Coincubation with 1 mM mevalonate or 5 $\mu$M GGPP, the immediate downstream metabolites of HMG-CoA reductase, significantly reversed the AICAR-induced enhancement of BMP-2 mRNA expression ($P < 0.05$) (Fig. 2C), although mevalonate or GGPP did not affect the expression of BMP-2 mRNA in the absence of AICAR (data not shown).

**Effect of AICAR on eNOS mRNA expression in osteoblastic MC3T3-E1 cells via the mevalonate pathway.** We examined whether or not AICAR could stimulate eNOS mRNA expression in osteoblastic MC3T3-E1 cells. AICAR significantly increased eNOS mRNA expression level at 0.5 mM after 7 days ($P < 0.05$) (Fig. 3A). Coincubation with 1 mM mevalonate or 5 $\mu$M GGPP significantly reversed the AICAR-induced enhancement of eNOS mRNA expression ($P < 0.05$) (Fig. 3B), although mevalonate or GGPP did not affect the expression of eNOS mRNA in the absence of AICAR (data not shown).

**Effects of an NOS inhibitor or a BMP antagonist on the AICAR-induced mineralization and OCN mRNA expression in osteoblastic MC3T3-E1 cells.** We investigated whether or not eNOS or BMP-2 were involved in the AICAR-induced reactions in MC3T3-E1 cells by inhibiting their activities. Coincubation with 1 mM l-NAME, an NOS inhibitor, or 100 ng/ml noggin, a BMP antagonist, significantly reversed the AICAR-induced enhancement of mineralization by both von Kossa and Alizarin red stainings after 21 days ($P < 0.05$) (Fig. 4, A and B) as well as that of OCN mRNA expression by real-time PCR after 7 days ($P < 0.05$) (Fig. 4C) in MC3T3-E1 cells.

**Effects of AICAR on the protein kinase B and ERK activation as well as BMP-2 and eNOS mRNA expression in osteoblastic MC3T3-E1 cells via the ERK pathway.** Western blot analysis revealed that 0.5 mM AICAR was able to phosphorylate protein kinase B (Akt) and ERK in MC3T3-E1 cells in time-dependent manners up for to 60 min (Fig. 5, A and B, respectively). Next, we examined whether or not AICAR could stimulate BMP-2 and eNOS mRNA expression in osteoblastic MC3T3-E1 cells via the activation of ERK. Coincubation with 25 $\mu$M PD-98059, an ERK inhibitor, significantly reversed the AICAR-induced enhancement of BMP-2 mRNA expression at 48 h ($P < 0.05$) (Fig. 5C) as well as enOS mRNA expression at 24 and 48 h ($P < 0.05$) (Fig. 5D).

**Effects of AICAR and fasudil on ROK activities in MC3T3-E1 cells.** To determine whether or not AICAR and fasudil, a specific ROK inhibitor, could suppress ROK activities in osteoblastic MC3T3-E1 cells, the cells were incubated with either 0.1–0.5 mM AICAR or $10^{-6}$ to $10^{-4}$ M fasudil in 2 h. Next, the protein was collected and analyzed as described in MATERIALS AND METHODS. Both AICAR (0.1 and 0.5 mM) and fasudil ($10^{-5}$ and $10^{-4}$ M) significantly suppressed the phosphorylation of ROK activities in MC3T3-E1 cells (Fig. 6A and B, respectively). Fasudil significantly suppressed the phosphorylation of ROK activities in MC3T3-E1 cells (Fig. 6B).

**Fig. 7. Effect of fasudil on alkaline phosphatase (ALP) activity in MC3T3-E1 cells.** A plate view of ALP staining (A) and quantification of ALP activity (B) in cultured MC3T3-E1 cells. ALP staining and activity were increased by fasudil ($10^{-4}$ M) compared with the control at 14 days. Results are expressed as the mean ± SE fold increase over control values ($n = 6$). *$P < 0.05$. The result was representative of three different experiments.
phorylation of the MSB, a ROK substrate \((P < 0.05)\) (Fig. 6, A and B, respectively).

**Effects of fasudil on the BMP-2 and eNOS mRNA expression, differentiation, and mineralization of MC3T3-E1 cells.** We examined whether or not fasudil could enhance the differentiation and mineralization of MC3T3-E1 cells. Fasudil significantly enhanced Alizarin red stainings (Fig. 7A) and Activities (Fig. 7B) at \(10^{-5}\) M after 14 days \((P < 0.001)\) and mineralization by von Kossa and Alizarin red stainings as well as Alizarin red quantification at \(10^{-3}\) and \(10^{-4}\) M after 21 days \((P < 0.01)\) (Fig. 8, A and B, respectively). Fasudil \((10^{-4}\) M\) significantly increased OCN mRNA expression in a time-dependent manner until 48 h (Fig. 8C). Fasudil significantly increased OCN mRNA expression at \(10^{-5}\) and \(10^{-4}\) M after 48 h by real-time PCR \((P < 0.01)\) (Fig. 8D). Moreover, we examined whether or not fasudil could enhance BMP-2 and eNOS mRNA expression in the cells. Fasudil \((10^{-4}\) M\) significantly increased the BMP-2 mRNA expression in a time-dependent manner until 48 h by real-time PCR \((P < 0.05)\) (Fig. 9A). Fasudil significantly increased BMP-2 expression at \(10^{-5}\) and \(10^{-4}\) M \((P < 0.05)\) (Fig. 9B), and fasudil also significantly increased eNOS mRNA expression at \(10^{-4}\) M after 48 h \((P < 0.05)\) (Fig. 9C).

**DISCUSSION**

In this study, we demonstrated that AMPK activation by AICAR augmented the mineralization and mRNA expression of eNOS, BMP-2, and OCN in osteoblastic MC3T3-E1 cells. These effects were reversed by the supplementation of mevalonate or GGPP, the immediate downstream metabolites of HMG-CoA reductase, suggesting that these effects were caused by the inhibition of HMG-CoA reductase. Coincubation with L-NAME, an NOS inhibitor, or noggin, a BMP-2 antagonist, significantly reversed the AICAR-induced increase in OCN mRNA expression as well as enhancement of mineralization. Thus activation of AMPK seems to promote the differentiation and mineralization of osteoblastic cells via inhibition of the mevalonate pathway and subsequent increases in eNOS and BMP-2 expression. Moreover, we showed that AICAR also activated the Akt and ERK signaling pathways and that AICAR-induced eNOS and BMP-2 mRNA expression was also reversed by PD-98059, an ERK inhibitor. These findings suggest that there are interplays between the AMPK pathway and the Akt and/or ERK pathways, and the AMPK and ERK pathways may act stimulatory on the osteoblastic cell function.

Statins are known to enhance bone formation by inhibiting HMG-CoA reductase in osteoblasts. Mundy et al. (30) showed that lovastatin and simvastatin increased bone formation when injected subcutaneously over the calvaria of mice and increased cancellous bone volume when orally administered to rats via increased expression of BMP-2. Thus agents inhibiting HMG-CoA reductase and/or its downstream molecules in osteoblasts have widely attracted attention as potential candidate drugs for the treatment of osteoporosis. Our findings suggest that medications that are able to activate AMPK would also have the ability to stimulate bone formation via inhibiting HMG-CoA reductase.

Several studies have shown that statins were able to activate Akt and to stabilize eNOS mRNA (10, 25, 26, 35). It was also documented that inhibition of ROK led to activation Akt/PKB, resulting in eNOS activation and an increase in nitric oxide production (23). Other studies have indicated that eNOS deficiency caused a significant reduction in bone mass in mice (1), whereas activation of Akt and nitric oxide stimulated BMP-2 transcription and osteoblast differentiation (10, 30). In the present study, we found that both AICAR and fasudil enhanced BMP-2 and eNOS mRNA expression through the inhibition of the mevalonate pathway and that an NOS inhibitor, L-NAME, partly but significantly reversed the AICAR-induced mineralization and OCN expression. Thus our study and others con-
firm that eNOS is a pivotal molecule that links the mevalonate pathway and osteoblast differentiation.

A couple of studies have revealed that there are interplays between the AMPK pathway and the MAPK pathway (19, 20), the latter of which is very important on the proliferation and differentiation of osteoblasts (16, 24). For instance, Kim et al. (22) have recently shown that AICAR activated ERK, a constituent of the MAPK pathway, and that ERK inhibitors abolished palmitate-induced apoptosis in human differentiated osteoblasts (22). In this study, we also found that AICAR phosphorylated Akt and ERK and that the AICAR-induced increases in BMP-2 and eNOS mRNA expression was reversed by PD-98059, an ERK inhibitor. These findings suggest that the MAPK pathway would act in concert with the AMPK pathway, thereby stimulating osteoblasts and enhancing bone formation.

In this study, we also showed that inhibition of ROK by fasudil induced BMP-2, eNOS, and OCN mRNA expression and enhanced mineralization in MC3T3-E1 cells. Moreover, we confirmed that fasudil and AICAR actually inhibited ROK activities in the cells by measuring them directly. Rho/ROK, which is activated by GGPP, is known to be located downstream of the mevalonate pathway. ROK is identified as one of the effectors of the small GTP-binding protein Rho. Accumulating evidence has demonstrated that the Rho/ROK pathway plays an important role in various cellular functions (34). At a molecular level, ROK upregulates various molecules that accelerate inflammation/oxidative stress and fibrosis, whereas it downregulates NOS. Harmey et al. (15) showed that activation of Rho by Pasteurella multocida toxin (PMT) resulted in increased proliferation but decreased differentiation of primary mouse calvarial osteoblasts, which exhibited reduced gene expression of osteoblastic markers, including RUNX2, ALP, type I collagen, and OCN, and reduced bone nodule formation. Conversely, they found that chemical inhibition of ROK prevented the effects of PMT and enhanced bone nodule formation in the absence of PMT. These effects correlated with altered BMP-2 and -4 expression. Ohnaka et al. (31) showed that inhibition of the mevalonate pathway by pitavastatin, one of lipophilic statins, could suppress lysophosphatidic acid-induced Rho/ROK activation, resulting in enhancement of BMP-2 and OCN mRNA expression in osteoblasts. Our results seem to be consistent with these studies, suggesting that the Rho/ROK pathway is an important negative regulator of osteoblast differentiation. In contrast, Meyers et al. (29) showed that overexpression of constitutively active RhoA enhanced the osteoblastic phenotype such as increased gene expression of type I collagen, ALP, and runt-related transcription factor 2, and suppressed the adipocytic phenotype of human mesenchymal stem cells cultured in modeled microgravity. Hence, it is possible that the role of Rho activity differs depending on the stage of osteoblastic differentiation.

Many clinical studies have shown that osteoporosis is associated with atherosclerosis or the cardiovascular disease. An epidemiological study indicated that aortic calcification was more prevalent in communities where osteoporosis was more prevalent (9). Other studies showed that, after age adjustment, calcified aortic plaques or aortic pulse wave velocity was inversely related to bone mineral density (BMD) of the lumbar spine or distal and proximal radius (7, 32, 37). Barenholz et al. (2) compared coronary calcium scores measured by electron beam computed tomography in asymptomatic, postmenopausal women with normal and low BMD, who were similar in age and body mass index, and found that the score was significantly higher in the osteoporosis group than in the control group. Moreover, low radial BMD was associated with increased risks of stroke (4) and cardiovascular death (38), after adjustments for age and several other potential confounders. We have previously shown that high low-density lipoprotein-cholesterol and low high-density lipoprotein-cholesterol are associated with low bone mass as well as atherosclerosis in postmenopausal women (39), suggesting that abnormalities in lipid metabolism might be the common factors underlying both osteoporosis and atherosclerosis. In this study, we also found that agents modulating AMPK and ROK activities might be candidate drugs not only curing hypercholesterolemia or the cardiovascular disease but also promoting bone formation for the treatment of osteoporosis. Thus biochemical observations and clinical ones strengthen the notion that osteoporosis is related to atherosclerosis or the cardiovascular disease.

In conclusion, we have established that inhibition of HMG-CoA reductase or ROK acts as an anabolic for bone and promotes the mineralization of osteoblasts by enhancing...
BMP-2 and eNOS expression. The present findings suggest that inhibition of the mevalonate pathway by activation of AMPK and inhibition of ROK simultaneously results in increasing bone mass as well as preventing atherosclerosis in the elderly person.

GRANTS

This study was partly supported by a grant from Shimane University.

REFERENCES


