# 学 位 論 文 の 要 旨

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学 位 論 文 名

Activation of AMP-kinase and Inhibition of Rho-kinase Induce the Mineralization of Osteoblastic MC3T3-E1 Cells Through Endothelial NOS and BMP-2 Expression

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## 論 文 内 容 の 要 旨

### INTRODUCTION

AMP-kinase (AMPK) is known to be expressed ubiquitously including bone, and could possibly influence bone metabolism. Recently, we have demonstrated for the first time that adiponectin and 5-aminoimidazole-4-carboxamide1-β-D-ribonucleoside (AICAR), both of which are able to activate AMPK, stimulated the differentiation and mineralization of osteoblastic MC3T3-E1 cells, 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, 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.

Statins are also known to affect bone, by inducing bone morphogenetic protein-2 (BMP-2) and endothelial nitric oxide synthase (eNOS) expression in osteoblasts and stimulating bone formation. Moreover, inhibition of Rho-kinase (ROK), which is located in the downstream of HMG-CoA reductase, by fasudil, a specific ROK inhibitor, has been shown to increase BMP-2 mRNA expression in human osteoblasts. 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.

### MATERIALS AND METHODS

*Cell culture*

MC3T3-E1 cells were cultured in α-MEM supplemented with 10% FBS and 1%

penicillin-streptomycin in 5% $CO_2$ at 37°C.

*Alkaline phosphatase staining*

Cultured cells were 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, 0.05 M $MgCl_2$, followed by incubation at room temperature for 6 hours in the dark.

*Assay of alkaline phosphatase activity*

The assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol, 1 mM $MgCl_2$, 8 mM p-nitrophenyl phosphate disodium, and cell homogenates. After a 4 min of incubation at 37°C, the reaction was stopped with 0.1N NaOH, and the absorbance was read at 405 nm. Each value was normalized to the protein concentration.

*Cell mineralization assay*

Mineralization of the cells was determined using von Kossa staining and Alizarin red staining after cells were grown in α-MEM supplemented with 10mM β-glycerophosphate for 2 weeks after reaching confluency.

*Real-time PCR quantification of gene expression*

Total RNA was isolated using Trisol reagent, and first-strand cDNA was synthesized using oligo-dT primer and SuperScript III cDNA synthesis kit. The cDNA was amplified using an ABI PRISM 7000 sequence detection system.

*Immunoblotting*

Cell lysates that contained an equivalent amount of protein were electrophoresed by 10% SDS-PAGE and transferred to nitrocellulose membrane. The blots were incubated overnight at 4°C with gentle shaking with Akt or ERK antibody. The blots were then washed, and the signal was visualized by chemiluminescence according to the manufacturer's protocol.

*Rho-kinase activity assay*

Rho-kinase activities were assessed by using the Rho-kinase assay kit as indicated by the manufacturer. Each value was normalized to the protein concentration.

*Statistical Analysis*

Results were expressed as mean ± SEM. Statistical evaluation was carried out with one-way analysis of variance (ANOVA). For all statistical tests, a value of $P<0.05$ was considered to indicate a statistically significant difference.

## RESULTS AND DISCUSSION

Real-time PCR and mineralization stainings revealed that AICAR significantly stimulated eNOS, BMP-2, and osteocalcin mRNA expression as well as mineralization in MC3T3-E1 cells. Supplementation of either mevalonate or geranyl-geranyl pyrophosphate (GGPP), the downstream molecules of HMG-CoA reductase, or co-incubation with either a NOS inhibitor, L-NAME, or a BMP-2 antagonist, noggin, significantly reversed these AICAR-induced reactions.

Western blot analysis showed that AICAR activated Akt and extracellular signal-regulated kinase (ERK). ERK inhibitor significantly reversed the AICAR-induced increase in eNOS and BMP-2

mRNA expression.

Measurement of ROK activities by ELISA revealed that both AICAR and fasudil significantly suppressed the phosphorylation of the myosin-binding subunit of myosin phosphate, a ROK substrate. Fasudil significantly enhanced eNOS, BMP-2, and osteocalcin mRNA expression as well as ALP activity and mineralization in the cells.

In this study, we demonstrated that AMPK activation by AICAR augmented the mineralization as well as the expression of eNOS, BMP-2, and OCN mRNA in MC3T3-E1 cells. These effects were reversed by the supplementation of mevalonate or GGPP. Co-incubation with a NOS inhibitor or a BMP-2 antagonist significantly reversed the AICAR-induced reactions. 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 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. Previous studies showed that statins 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 attentions 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 inhibition of HMG-CoA reductase was able to activate Akt and to stabilize eNOS mRNA. It was also documented that inhibition of ROK led to activation Akt/PKB, resulting in eNOS activation and an increase in NO production. Previous studies have indicated that eNOS deficiency caused a significant reduction in bone mass in mice, while activation of Akt and NO stimulated BMP-2 transcription and osteoblast differentiation. In the present study, we found that both of AICAR and fasudil enhanced BMP-2 and eNOS mRNA expression through the inhibition of the mevalonate pathway, and that a NOS inhibitor partly but significantly reversed the AICAR-induced mineralization as well as OCN expression. Thus, our study and others confirm that eNOS is a pivotal molecule that links the mevalonate pathway and osteoblast differentiation.

## CONCLUSION

We have established that inhibition of HMG-CoA reductase or ROK acts as 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. Thus, these agents could be candidate drugs that promote bone formation for the treatment of osteoporosis.