学 位 論 文 の 要 旨

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学　位　論　文　名　Activation of AMP-activated Protein Kinase Decreases Receptor Activator of NF-kB Ligand Expression And Increases Sclerostin Expression by Inhibiting The Mevalonate Pathway in Osteocytic MLO-Y4 Cells

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論 文 内 容 の 要 旨

INTRODUCTION

Bone tissue is constantly renewed by a balanced between bone formation and bone resorption. Osteocytes regulate bone resorption by regulating the expression of receptor activator of nuclear factor-kB ligand (RANKL), which induces osteoclastogenesis, and osteoprotegerin (OPG), a decoy receptor for RANKL. Osteocytes also produce sclerostin, a protein encoded by *Sost*, that inhibits osteoblast activity and bone formation.

AMP-activated protein kinase (AMPK) is a crucial regulator of energy and metabolic homeostasis. It is a heterotrimeric complex containing a catalytic α subunit and regulatory β and γ subunits. When cellular AMP/ATP ratio increases, AMPK is activated through the phosphorylation of α subunit. Once activated, AMPK inactivates several metabolic enzymes involved in ATP-consuming cellular events, including cholesterol and protein synthesis, by inhibiting HMG-CoA reductase. Previous studies have shown that AMPK subunits are expressed in bone tissue and that AMPK plays important roles in bone metabolism. Mice lacking the AMPKα1 subunit experienced a significant reduction in bone mass, suggesting that this subunit plays a major role in skeletal metabolism. Several *in vitro* studies showed that activated AMPK inhibits osteoclast formation and bone resorption. Moreover, we previously demonstrated that AMPK activation stimulated the differentiation and mineralization of osteoblastic MC3T3-E1 cells by inhibiting mevalonate pathway. However, the roles of AMPK in the function of

osteocytes are still unclear. Thus, this study aimed to investigate the effects of AMPK activation on RANKL, OPG, and sclerostin expression in osteocytic MLO-Y4 cells.

MATERIALS AND METHODS

We used MLO-Y4 cells, a murine long bone-derived osteocytic cell line, and the cells were cultured on collagen-coated plates in α-minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in 5% CO_2 at 37°C. After reaching confluence, the cells were treated with various reagents. We assessed expression and phosphorylation of the AMPKα1 subunit in MLO-Y4 cells by reverse transcription (RT)-PCR and Western blot. To examine the expressions of *Rankl*, *Opg*, and *Sost*, quantitative real-time PCR using SYBR green chemistry was performed. *36B4*, a housekeeping gene, was used to normalize the differences in the efficiencies of RT. Total RNA was extracted from the cultured cells using Trizol reagent and used for synthesizing single-stranded cDNA. For Western blot analysis, the cell lysates were collected and electrophoresed by performing SDS-PAGE on 10% polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4 °C with a specific antibody. After washed, the membrane was incubated with a secondary antibody for 30 min at 4 °C. The signals were visualized using an enhanced chemiluminescence technique. RNA interference was used to down-regulate the expression of AMPKα subunit in MLO-Y4 cells. Small interfering RNA (siRNA) and reagents for AMPKα1, AMPKα2 and nonspecific control siRNA duplexes were designed and synthesized by Customer SMARTpool siRNA Design from Dharmacon (Lafayette, CO, USA).

RESULTS AND DISCUSSION

We first confirmed the mRNA expression of the AMPKα1 and AMPKα2 subunits. Moreover, the protein levels of AMPKα1 and AMPKα2 subunits were examined in mouse stromal ST2, mouse osteoblast-like MC3T3-E1, and MLO-Y4 cells. The protein expression of AMPKα2 was relatively low in MLO-Y4 cells compared to other cells. We also confirmed that treatment with 1.0 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleotide (AICAR), an activator of AMPK, phosphorylated AMPKα subunit until 3 h.

Next, we examined effects of AMPK activation on the mRNA expression of *Rankl*, *Opg*, and *Sost* in MLO-Y4 cells. Real-time PCR showed that AICAR significantly decreased *Rankl* expression and *Rankl/Opg* ratio in a dose-dependent manner after 48 h treatment with AICAR but did not affect *Opg* expression. In contrast, AICAR treatment significantly increased *Sost*

expression in a dose-dependent manner. Moreover, we examined the time-dependent effects of AICAR during 48 h treatment. We observed that *Rankl* expression and *Rankl/Opg* ratio were significantly decreased in a time-dependent manner during 48 h and that *Sost* expression peaked after 24 h treatment. However, AICAR treatment did not affect *Opg* expression at any time point. Western blotting showed that 72 h treatment with AICAR suppressed RANKL protein expression in a dose-dependent manner and increased sclerostin expression. Then, to examine the effects of AMPKα subunits knockdown on MLO-Y4 cells, we investigated the expression of *Rankl* and *Sost* in the siRNA-transfected cells. The total RNA was collected on 4 days after the siRNA treatment. Real-time PCR showed increased *Rankl* expression by the siRNA-AMPKα1, but not siRNA-AMPKα2. On the other hand, the expression of *Sost* was not significantly affected by the siRNA-AMPKα1 or siRNA-AMPKα2. Taken together, these findings suggest that AMPK activation inhibits osteoclast activity by decreasing RANKL expression in osteocytes and negatively affects osteoblasts by increasing sclerostin expression. However, the effect of AICAR on the increased *Sost* mRNA was temporal, and the mRNA expression was not changed by knockdown of AMPKα, suggesting that the role of AMPK in regulation of sclerostin expression may be trivial.

To examine whether the mevalonate pathway was involved in the effects of AMPK activation, we investigated the effects of simvastatin on the expression of *Rankl* and *Sost.* Real-time PCR showed that treatment of MLO-Y4 cells with 1.0 μM simvastatin significantly decreased *Rankl* expression and increased *Sost* expression, which was similar to those observed after treatment with 0.5-1.0 mM AICAR. Moreover, co-incubation with 1.0 mM mevalonate or 5.0 μM geranylgeranyl pyrophosphate (GGPP), the immediate downstream metabolites of HMG-CoA reductase, significantly reversed AICAR-suppressed *Rankl* expression and AICAR-augmented *Sost* expression. However, mevalonate or GGPP did not affect *Rankl* or *Sost* expression in the absence of AICAR. These findings suggest that the mevalonate pathway plays important roles in regulating bone remodeling and that AMPK activation decreases RANKL expression and increases sclerostin expression by inhibiting HMG-CoA reductase in osteocytes.

CONCLUSION

The present study showed that AMPK activation decreased RANKL expression and increased sclerostin expression by inhibiting the mevalonate pathway in osteocytic MLO-Y4 cells. Further studies on the role of AMPK in osteocytes would provide new insights on the effects of AMPK on bone metabolism.