Activation of AMP-activated protein kinase decreases receptor activator of NF-κB ligand expression and increases sclerostin expression by inhibiting the mevalonate pathway in osteocytic MLO-Y4 cells

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ABSTRACT

Background: AMP-activated protein kinase (AMPK) plays important roles in bone metabolism; however, little is known about its role in osteocytes. This study investigated the effects of AMPK activation on the expression of receptor activator of NF-κB ligand (RANKL) and sclerostin in osteocytes.
Results: Real-time PCR showed that AMPK activation by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) significantly decreased the expression of *Rankl* in a dose- and time-dependent manner and significantly increased the expression of *Sost*, the gene encoding sclerostin, in osteocytic MLO-Y4 cells. Western blotting confirmed that AICAR decreased RANKL protein levels and increased sclerostin levels. In addition, suppression of AMPKα1 by siRNA significantly increased the expression of *Rankl* on 4 days after the transfection of siRNA, while *Sost* expression was not changed. Simvastatin, an inhibitor of HMG-CoA reductase, significantly decreased *Rankl* expression and increased *Sost* expression in MLO-Y4 cells. Supplementation with mevalonate or geranylgeranyl pyrophosphate, which are downstream metabolites of HMG-CoA reductase, significantly reversed the effects of AICAR.
Conclusion: These findings indicated that AMPK regulated RANKL and sclerostin expression through the mevalonate pathway in osteocytes.

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1. Introduction

Bone tissue is constantly renewed by a balanced between bone formation and bone resorption. Several studies have shown that osteocytes play multifunctional roles in orchestrating bone remodeling by regulating both osteoblast and osteoclast functions [1,2]. A recent study showed that osteocytes expressed much higher levels of receptor activator of nuclear factor-κB ligand (RANKL) and had a great capacity to support osteoclastogenesis [3]. Previous studies have indicated that osteocyte-derived RANKL plays a key role in bone remodeling in response to mechanical loading [3–5]. Thus, osteocytes are the main cells involved in the initiation of bone remodeling. In addition, osteocytes produce osteoprotegerin (OPG), a decoy receptor for RANKL. Thus, osteocytes regulate bone resorption by regulating RANKL/OPG ratio [2]. Osteocytes also produce sclerostin, a protein encoded by *Sost*, that inhibits osteoblast activity by blocking Wnt/beta-catenin pathway [6,7].

AMP-activated protein kinase (AMPK) is a crucial regulator of energy and metabolic homeostasis at the cellular and whole-organism levels [8,9]. AMPK is a heterotrimeric complex containing a catalytic α subunit and regulatory β and γ subunits and functions as a serine/threonine kinase [10]. An increase in cellular AMP/ATP ratio activates AMPK through the phosphorylation of the α subunit (Thr 172). Once activated, AMPK inactivates several metabolic enzymes involved in ATP-consuming cellular events, including cholesterol and protein synthesis, by inhibiting HMG-CoA reductase [11].

Increasing evidence indicates that osteoporosis is a disorder of energy metabolism. Recent studies have shown that the AMPK

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signaling pathway plays pivotal roles in bone physiology [12]. AMPK subunits are expressed in bone tissue and cells, with AMPKα1 subunit being the dominant catalytic isoform expressed in the bone [13]. A study showed that mice lacking the AMPKα1 subunit (AMPKα1$^{-/-}$ mice) experienced a significant reduction in bone mass [14], suggesting that this subunit played a major role in skeletal metabolism. Activated AMPK inhibits osteoclast formation and bone resorption in vitro [15]. We previously showed that AMPK activation stimulated the differentiation and mineralization of osteoblastic MC3T3-E1 cells by inhibiting mevalonate pathway [16—18]. Moreover, we recently reported that AMPK activation exerted protective effects against homocysteine-induced apoptosis of osteocytic MLO-Y4 cells [19].

However, the effects of AMPK activation on RANKL and sclerostin expression in osteocytes are unclear. This is the first study to show that AMPK activation by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) decreased RANKL expression and increased sclerostin expression by inhibiting the mevalonate pathway in osteocytic MLO-Y4 cells.

2. Materials and methods

2.1. Reagents

Cell culture medium and supplements were purchased from Gibco-BRL (Rockville, MD). AICAR and antibodies against total AMPKα and phosphorylated AMPKα were purchased from Cell Signaling (Beverly, MA). Antibodies against AMPKα1 and α2 subunits were purchased from Abcam (Tokyo, Japan). Simvastatin, mevalonate, and geranylgeranyl pyrophosphate (GGPP) were purchased from Sigma—Aldrich (St. Louis, MO). Antibodies against RANKL and sclerostin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Abcam, respectively. Rabbit monoclonal antibodies were purchased from Sigma—Aldrich. All other chemicals were of the highest grade available commercially.

2.2. Cell cultures

MLO-Y4 cell line, a murine long bone-derived osteocytic cell line, was kindly provided by Dr. Lynda F. Bonewald. MLO-Y4 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. The medium was changed twice a week, and the cells were passaged after they reached 80% confluency.

2.3. Reverse transcription—PCR to identify the AMPKα1 subunit

The mRNA expression of the AMPKα1 subunit in MLO-Y4 cells was determined by performing reverse transcription (RT)—PCR. Total RNA was extracted from the cultured MLO-Y4 cells by using TRIzol reagent (Invitrogen, San Diego, CA), according to the manufacturer's recommended protocol. In all, 2 μg of the total RNA was used for synthesizing single-stranded cDNA (cDNA synthesis kit; Invitrogen). PCR conditions were as follows: 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C, and elongation at 72 °C for 1 min. PCR products were electrophoresed on a 1.8% agarose gel stained with ethidium bromide and were visualized under ultraviolet (UV) light by using an electronic UV transilluminator (Toyobo Co. Ltd., Tokyo, Japan).

2.4. Quantification of gene expression by performing real-time PCR

SYBR green chemistry was used to determine the mRNA levels of *Rankl*, *Opg*, *Sost*, and *36B4*, a housekeeping gene. The following primers were used: *Rankl* forward, 5′-CACCATCAGCTGAAGATAGT-3′ and *Rankl* reverse, 5′-CCAAGATCTCTAACATGACG-3′; *Opg* forward, 5′-AGCTGCTGAAGCTGTGGAA-3′ and *Opg* reverse, 5′-TGTTCGAGTGGCCGAGAT-3′; *Sost* forward, 5′-GGAATGATGCCACA-GAGGTCAT-3′ and *Sost* reverse, 5′-CCCGGTTCATGGTCTGGTT-3′; and *36B4* forward, 5′-AAGCGCGTCCTGGCATTGTCT-3′ and *36B4* reverse, 5′-CCGCAGGGGCAGCAGTGGT-3′. Real-time PCR was performed in a 25-μL reaction mixture containing 1 μL cDNA by using ABI PRISM 7000 (Applied Biosystems, Waltham, MA). Double-stranded DNA-specific SYBR Green I was mixed with PCR buffer provided in SYBR Green Real-Time PCR Master Mix (Toyobo Co. Ltd.) to quantify the PCR products. PCR conditions were as follows: initial denaturation at 95 °C for 15 min and 40 cycles of denaturation at 94 °C for 15 s and annealing and extension at 60 °C for 1 min. The mRNA level of *36B4* was used to normalize the differences in the efficiency of RT.

2.5. Western blotting

For western blotting, the cells were plated in 6-well plates and were cultured as described above. After reaching confluency, the cells were treated with each agent for 48 h. The cells were then rinsed with ice-cold PBS and were scraped on ice in lysis buffer (65.8 mM Tris—HCl [pH 6.8], 26.3% [w/v] glycerol, 2.1% SDS, and 0.01% bromophenol blue; Bio-Rad, Hercules, CA) supplemented with 2-mercaptoethanol at a final concentration of 5%. The cell lysates were sonicated for 20 s and were electrophoresed by performing SDS-PAGE on a 10% polyacrylamide gel. The separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked with TBS containing 1% Tween 20 (Bio-Rad) and 3% bovine serum albumin for 1 h at 4 °C and was incubated overnight with specific antibodies at 4 °C with gentle shaking. The membrane was then extensively washed with TBS containing 1% Tween 20 and was incubated with horseradish peroxidase-coupled rabbit anti-mouse antibody in TBS for 30 min at 4 °C. The membrane was washed, and signals were detected using an enhanced chemiluminescence technique.

2.6. RNA interference for AMPKα subunits

RNA interference was used to down-regulate the expression of AMPKα subunit in MLO-Y4 cells. SMARTpool small interfering RNA (siRNA) and SMARTpool reagents for AMPKα1, AMPKα2 and nonspecific control siRNA duplexes were designed and synthesized by Customer SMARTpool siRNA Design from Dharmacon (Lafayette, CO). For gene knock down experiments, MLO-Y4 cells were plated in 6 cm dish and cultured for 48 h in α-MEM containing 10% FBS and antibiotics. Next, after 24 h incubation in medium without antibiotics, cells were transfected with siRNAs (50 nM) using transfection reagent according to the manufacture's instructions. After another 48 h of culture, cells were recultured in another in α-MEM containing 10% FBS and antibiotics.

2.7. Statistical analysis

Results are expressed as mean ± standard error (SE). Statistical differences between groups were determined using one-way ANOVA followed by Fisher's protected least significant difference. For all statistical tests, a *p value of* <0.05 was considered statistically significant.