Molecular and Cellular Endocrinology 339 (2011) 114–119

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

AMP-activated protein kinase attenuates Wnt/β-catenin signaling in human osteoblastic Saos-2 cells

Tomozumi Takatani*, Masanori Minagawa, Rieko Takatani, Kaori Kinoshita, Yoichi Kohno

Department of Pediatrics, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba 260-8670, Japan

ARTICLE INFO

Article history:
Received 26 November 2010
Received in revised form 28 March 2011
Accepted 1 April 2011

Keywords:
AMPK
Metformin
Wnt
β-Catenin
Osteoblast

ABSTRACT

AMP-activated protein kinase (AMPK) is a key sensor of cellular energetic conditions. Recent studies suggest that AMPK affects osteoblast differentiation, although its role and mechanism are not fully understood. One of the most important signals in osteoblast differentiation is the Wnt/β-catenin pathway which induces T-cell transcription factor 1 (TCF)-dependent transcription. Using human osteoblast-like Saos-2 cells, we determined whether AMPK modulates Wnt/β-catenin signaling in osteoblasts. Chemical activators of AMPK (AICAR [5-aminoimidazole-4-carboxamide riboside], metformin) suppressed Wnt3a-induced TCF-dependent transcriptional activity. Transactivation by Wnt was potentiated by inhibiting β-catenin degradation with lithium chloride (LiCl). LiCl-induced Wnt transactivation was suppressed by addition of metformin. Metformin increased the phosphorylation of β-catenin and decreased β-catenin protein levels leading to suppression of Wnt/β-catenin signaling. Our present study showed that AMPK attenuates Wnt/β-catenin signaling by reducing β-catenin protein levels in osteoblast-like cells.

1. Introduction

AMP-activated protein kinase (AMPK) is an energy sensing serine/threonine kinase protein complex that controls energy balance within cells (Kahn et al., 2005; Kemp et al., 2003; Steinberg and Kemp, 2009). It is involved in the regulation of glucose and lipid metabolism, mitochondrial function, and protein synthesis (Kahn et al., 2005; Kemp et al., 2003; Steinberg and Kemp, 2009), and has non-metabolic roles in the control of cell polarity, division, viability, and growth (Shaw, 2009; Zhang et al., 2006; Zheng and Cantley, 2007).

AMPK is a heterotrimeric complex containing a catalytic α-subunit and regulatory β- and γ-subunits (Mitchelhill et al., 1994; Stapleton et al., 1994). There are seven known subunit isoforms including α1 and α2 (Stapleton et al., 1996), β1 and β2 (Chen et al., 1999), and γ1, γ2, and γ3 (Cheung et al., 2000); all their combinations give rise to a large variety of heterotrimeric complexes.

AMPK is expressed ubiquitously and novel AMPK functions have been extensively studied in various organs. In bone, AMPK is a potential regulator of bone and energy metabolism, and may represent an important therapeutic target for the treatment of detrimental skeletal defects such as those associated with obesity. Presently, knowledge of the function and regulation of AMPK in bone are limited. Conflicting reports suggest that AMPK activation either inhibits terminal osteoblast differentiation due to matrix mineralization and decreased expression of osteoblast differentiation markers (Kasai et al., 2009), or stimulates osteoblast differentiation (Cortizo et al., 2006; Kanazawa et al., 2008). These data indicate that the roles of AMPK in bone metabolism, osteoblast differentiation, and the regulation of bone mass and remodeling are unclear.

One of the most important pathways leading to osteoblast differentiation during bone formation involves Wnt signaling (Macsai et al., 2008; Piters et al., 2008). Wnt proteins are secreted glycoproteins that stimulate receptor-mediated signal transduction pathways to control various cellular activities. At least three different signaling pathways are activated by Wnt (Strutt, 2003). Of these, the Wnt/β-catenin signaling pathway directs bone metabolism. This pathway is triggered when Wnt binds to a receptor complex comprising a frizzled protein and a low-density lipoprotein receptor-related protein (LRP), LRP 5/6. The binding of Wnt to its receptor activates the disheveled protein thereby inhibiting

Abbreviations: AMPK, AMP-activated protein kinase; TCF, T-cell transcription factor; LiCl, lithium chloride; LRP, low-density lipoprotein receptor-related protein; APC, adenomatous polyposis coli; axin, axis inhibitor; CK1, casein kinase 1; GSK3β, glycogen synthase kinase-3-beta; araA, 9-beta-arabinofuranosyl adenine; AICAR, 5-aminoimidazole-4-carboxamide riboside; CM, conditioned media; L Wnt3a cells, Wnt3a-producing mouse L cells; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun-kinase; TGF-β, transforming growth factor-beta; BMP, bone morphogenic protein.

* Corresponding author. Tel.: +81 43 226 2144; fax: +81 43 226 2145.
E-mail address: ttgjun@yahoo.co.jp (T. Takatani).

0303-7207/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2011.04.003

β-catenin destruction complexes containing adenomatous polyposis coli (APC) and axis inhibitor (axin). In the absence of Wnt binding, β-catenin is phosphorylated by casein kinase 1 (CK1) and glycogen synthase kinase-3-beta (GSK3β), and degraded through the ubiquitin–proteasome pathway. Thus, ligand–receptor binding leads to the accumulation of β-catenin and its entry into the nucleus where it interacts with T-cell factor (TCF) and activates transcription of Wnt target genes (Hartmann, 2006).

Evidence suggests that the Wnt/β-catenin signaling pathway plays a central role in bone metabolism. Recent reports indicate that LRP5- and LRP6-deficent mice possess reduced bone mass (Holmen et al., 2004; Kato et al., 2002); that LRP-5 gain-of-function mice display high bone mass due to a reduction of apoptosis and an increased number of osteoblasts (Babij et al., 2003); that β-catenin conditional knockout mice develop osteopenia as changes in the expression of osteoprotegerin block osteoblast differentiation (Glass et al., 2005); and that inactivation of β-catenin at the early progenitor stage prevents osteoblast differentiation (Day et al., 2005).

Considering the potential role of AMPK in bone metabolism and the crucial effects of Wnt/β-catenin signaling on osteoblast differentiation, we hypothesized that AMPK affects osteoblast function by modulating the Wnt/β-catenin signaling pathway. In this study, we examined the effect of AMPK on the Wnt/β-catenin signaling pathway in human osteoblast-like Saos-2 cells. We found that AMPK activation suppressed Wnt/β-catenin signals via diminished β-catenin levels. This crosstalk between AMPK and the Wnt/β-catenin signaling pathway may be important for understanding the effect of AMPK on osteoblast function *in vitro* and bone remodeling *in vivo*.

2. Materials and methods

2.1. Materials

Metformin (1,1-dimethylbiguanide), araA (9-beta-arabinofuranosyl adenine), and AICAR (5-aminoimidazole-4-carboxamide riboside) were purchased from Sigma–Aldrich, Inc., (St. Louis, MO); LiCl was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Other materials and chemicals were purchased from commercial sources.

2.2. Cell culture and plasmid construction

The human osteoblastic cell line Saos-2 was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Super(8x)TOP-Flash containing TCF-binding sites and Super(8x)FOP-Flash with mutated TCF-binding sites were provided by R.T. Moon (Veeman et al., 2003). pcDNA3-β-catenin mutants (S33Y) were supplied by E.R. Fearon (Kolligs et al., 1999). A control *Renilla* luciferase plasmid phRL-TK was purchased from Promega (Madison, WI).

2.3. Preparation of Wnt3a-containing conditioned medium

Wnt3a-producing mouse L cells (L Wnt3a cells) and control L cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Wnt3a and control conditioned media (CM) were collected according to the protocol provided by ATCC.

2.4. Reporter assay

To evaluate TCF-dependent transcriptional activity, Saos-2 cells were seeded in 12-well plates and transfected in triplicate with Super(8x)TOP-Flash or Super(8x)FOP-Flash, and phRL-TK. FuGene HD Transfection reagent (Roche Diagnostics, Mannheim, Germany) was used for transient transfections. Appropriate stimulants were added 24 h after transfection. Cell lysates were harvested and reporter activities were measured with the Dual-Luciferase Reporter Assay system (Promega, Madison, WI). The firefly luciferase activities obtained from Super(8x)TOP-Flash and Super(8x)FOP-Flash were normalized to *Renilla* luciferase activities.

2.5. Western blotting

Cells were harvested and suspended in radioimmunoprecipitation assay lysis buffer (50 mM Tris–HCl [pH7.6], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and a phosphatase inhibitor cocktail (Nacalai Tesque). After incubation on ice for 15 min, cell lysates were centrifuged at 14,000 rpm for 15 min at 4 °C. The protein contents of the supernatants were determined with the Bradford ULTRA Total Protein Quantitation Kit (Novexin Ltd., Cambridge, UK). Proteins were separated via 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Tefco, Tokyo, Japan). Membranes were blocked with Blocking One (Nacalai Tesque) for non-phosphorylated proteins or Blocking One-P (Nacalai Tesque) for phosphorylated proteins for 1 h at room temperature, followed by incubation with various primary antibodies including rabbit anti-AMPKα (1:1000; Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-AMPKα (Thr172) (1:1,000; Cell Signaling Technology), rabbit anti-β-catenin (1:10,000; Epitomics, Burlingame, CA), rabbit anti-phospho-β-catenin (Ser33/37/Thr41) (1:1000; Cell Signaling Technology), and mouse anti-β-actin (1:2,000; Abcam, Cambridge, UK) overnight at 4 °C. After three washes in 0.1% Tween 20 in Tris-buffered saline (TBS), membranes were incubated with anti-rabbit IgG horseradish peroxidase conjugated secondary antibodies (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-mouse IgG horseradish peroxidase conjugated secondary antibodies (1:2500; Santa Cruz Biotechnology) for 1 h at room temperature. After three washes in 0.1% Tween 20 in TBS, peroxidase activity signals on PVDF membranes were detected with the WEST-one™ western blot detection system (iNtRON Biotechnology, Seoul, Korea) according to the manufacture's protocol. Densitometric measurements of immunoreactive bands on films were quantified with Image J software (Windows version; National Institutes of Health).

2.6. Real-time RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA (1.0 μg) was reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan). Real-time RT-PCR was carried out using the Chromo4 PCR system (Bio-Rad Laboratories, Hercules, CA) with FastStart Universal SYBR Green Master (Roche Diagnostics). The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min. The specific primer sequences were: β-catenin: forward, 5′-TTCTGGTGCCACTACCACAGC-3′, reverse, 5′-TGCATGCCCTCATCTAATGTC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5′-CAGTCAACGGATTTGGTCGT-3′, reverse, 5′-GACAAGCTTCCCGTTCTCAG-3′. GAPDH was used to normalize differences in RNA isolation, RNA degradation, and reverse transcription efficiency.

2.7. Statistical analysis

All data are presented as means ± standard deviations. Each experiment was repeated three times and the results obtained were similar to one another. The differences between two groups were assessed using Student's *t*-test and the differences between three or more groups were evaluated with one-way ANOVA, followed by the Bonferroni method for multiple comparisons between pairs. Statistical analyses were carried out with Dr. SPSS II (SPSS Japan Inc., Tokyo, Japan). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Metformin induced and maintained AMPKα phosphorylation in Saos-2 cells

We evaluated the effect of the AMPK activator, metformin on the phosphorylation of the AMPKα subunits in Saos-2 cells (Fig. 1A). Levels of phosphorylated AMPKα were significantly increased after 24-h stimulation with 2.0 mM metformin (Fig. 1B, left) and remained elevated at 48 h (Fig. 1B, right).

3.2. Metformin inhibited TCF-dependent transactivation of Wnt3a

To determine whether AMPK activation affects the Wnt/β-catenin signaling pathway, cells were transfected with Super(8x)TOP-Flash or the control Super(8x)FOP-Flash, and TCF-dependent transcriptional activities were measured. Super(8x)FOP-Flash reporter activity was not affected by metformin (Fig. 2A), but Wnt3a CM-induced Super(8x)TOP-Flash reporter activity was significantly suppressed in a dose-dependent manner (Fig. 2B).