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AMP-activated protein kinase (AMPK) activation regulates *in vitro* bone formation and bone mass

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Abstract

Adenosine 5'-monophosphate-activated protein kinase (AMPK), a regulator of energy homeostasis, has a central role in mediating the appetite-modulating and metabolic effects of many hormones and antidiabetic drugs metformin and glitazones. The objective of this study was to determine if AMPK can be activated in osteoblasts by known AMPK modulators and if AMPK activity is involved in osteoblast function *in vitro* and regulation of bone mass *in vivo*. ROS 17/2.8 rat osteoblast-like cells were cultured in the presence of AMPK activators (AICAR and metformin), AMPK inhibitor (compound C), the gastric peptide hormone ghrelin and the beta-adrenergic blocker propranolol. AMPK activity was measured in cell lysates by a functional kinase assay and AMPK protein phosphorylation was studied by Western Blotting using an antibody recognizing AMPK Thr-172 residue. We demonstrated that treatment of ROS 17/2.8 cells with AICAR and metformin stimulates Thr-172 phosphorylation of AMPK and dose-dependently increases its activity. In contrast, treatment of ROS 17/2.8 cells with compound C inhibited AMPK phosphorylation. Ghrelin and propranolol dose-dependently increased AMPK phosphorylation and activity. Cell proliferation and alkaline phosphatase activity were not affected by metformin treatment while AICAR significantly inhibited ROS 17/2.8 cell proliferation and alkaline phosphatase activity at high concentrations. To study the effect of AMPK activation on bone formation *in vitro*, primary osteoblasts obtained from rat calvaria were cultured for 14–17 days in the presence of AICAR, metformin and compound C. Formation of 'trabecular-shaped' bone nodules was evaluated following alizarin red staining. We demonstrated that both AICAR and metformin dose-dependently increase trabecular bone nodule formation, while compound C inhibits bone formation. When primary osteoblasts were co-treated with AICAR and compound C, compound C suppressed the stimulatory effect of AICAR on bone nodule formation. AMPK is a $\alpha\beta\gamma$ heterotrimer, where α is the catalytic subunit. RT-PCR analysis of AMPK subunits in ROS17/2.8 osteoblastic cells and in mouse tibia showed that the AMPK α 1 subunit is the dominant isoform expressed in bone. We analysed the bone phenotype of 4 month-old male wild type (WT) and AMPK α 1 $^{-/-}$ KO mice using micro-CT. Both cortical and trabecular bone compartments were smaller in the AMPK α 1-deficient mice compared to the WT mice. Altogether, our data support a role for AMPK signalling in skeletal physiology.

Keywords

AMP kinase; Osteoblasts; Knockout mice; Energy metabolism; Bone; Metformin

Introduction

Recent evidence for a direct hormonal link between bone remodelling, food intake and glucose metabolism has brought back into the spotlight the control of bone mass and its relationship to energy homeostasis. While communication between fat and bone was debated for a long time, new evidence has emerged with the discovery that the adipocyte-secreted hormone leptin inhibits bone formation through hypothalamic and sympathetic nervous system (SNS) relays [1,2]. The latest demonstration of a feedback control by the skeleton of glucose and fat metabolism through the systemic release of the osteoblast-specific protein osteocalcin [3] has substantiated the reciprocal relationship between bone and energy metabolism. This link is also suggested by the close association between obesity, osteoporosis and diabetes [4,5]. Loss of body weight is indeed associated with bone loss and both obesity and osteoporosis share a common progenitor cell, the mesenchymal stem cell. Diabetes mellitus is a well known secondary cause of osteoporosis.

An essential mediator of the central and peripheral effects of many appetite-regulating hormones including leptin is adenosine 5'-monophosphate-activated protein kinase (AMPK). AMPK has emerged over the last decade as a key sensing mechanism in the regulation of cellular energy homeostasis [for reviews, see 6-9]. It is a highly conserved serine/threonine Heterotrimeric protein consisting of a catalytic α subunit and two regulatory β and γ subunits [10]. Several isoforms exist for each of these subunits and all their combinations give rise to 12 different complexes. AMPK senses the AMP/ATP ratio within the cell, and once activated, switches on catabolic pathways (energy generating) and switches off anabolic pathways (energy consuming). AMPK is activated allosterically by AMP which binds the γ subunit, and by its upstream kinases promoting phosphorylation of Thr 172 within the α catalytic subunit. To date, there are three known AMPK kinases, the tumour suppressor kinase LKB1, calmodulin kinase kinase (CaMKK), or transforming growth factor-beta-activated kinase (TAK1) [10-12]. Recent data demonstrate that AMPK activity can also be modulated independently of AMP and phosphorylation by other agents such as the cell-death-inducing like-effector A (Cide-A) which mediates AMPK ubiquitination and degradation [13], and kinase suppressor of Ras 2 (KSR2) which interacts with AMPK [14]. The latest identification of a glycogen-binding domain in the β subunit of AMPK suggests that AMPK may also act as a glycogen sensor [15]. AMPK regulates many metabolic pathways in peripheral tissues by phosphorylating metabolic enzymes and affecting expression of genes involved in energy metabolism, cell signalling, cell proliferation and differentiation, apoptosis, immunity, and vascularisation [9,16-21]. It mediates the appetite-modulating and metabolic effects of many hormones and neuromodulators on appetite, lipid and glucose metabolisms [7,22-25]. In addition to its effect on energy balance at the cellular level, AMPK also plays a key role in the control of whole body energy homeostasis by integrating, at the hypothalamic level, nutrient and hormonal signals that regulate food intake and energy expenditure [8,26].

AMPK is expressed ubiquitously but its function and regulation in bone have been poorly investigated. It was recently demonstrated that the AMPK activator AICAR (5-aminoimidazole-4-carboxamide-1- β -D-ribose nucleoside) stimulates proliferation, differentiation and mineralisation of osteoblastic MC3T3-E1 cells via inhibition of the mevalonate pathway and subsequent increase in endothelial nitric oxide synthase [eNOS] [27,28]. However, this is apparently in contrast with recent findings from Kasai et al. [29]

which demonstrate that osteoblast differentiation is functionally associated with decreased AMPK activity. AMPK activity has also been shown to be involved in the increase in cyclooxygenase-2 (COX-2) expression in osteoblasts in response to ultrasound [30] as well as in the protection of osteoblast apoptosis induced by the saturated fatty acid palmitate [31]. Overall, those studies suggest that AMPK signalling may play a role in bone metabolism. Interestingly, many hormones and neuromediators, including leptin, ghrelin, cannabinoids and the SNS, that regulate food intake and energy expenditure through AMPK activation, also regulate bone mass either by directly binding to their respective receptors expressed by bone cells or via central hypothalamic pathways [1,2,32-38]. AMPK is also involved in the mechanism of action of two commonly used antidiabetic drugs, metformin and thiazolidinediones (TZDs) [39,40]. Both drugs affect bone cell function *in vitro* [41-44] and TZDs have recently been shown to have detrimental skeletal effects [45,46].

The aim of our study was to investigate whether AMPK can be activated in osteoblasts by known AMPK modulators as well as by hormones and drugs that regulate body weight and bone mass, such as ghrelin and the β -blocker propranolol, and if AMPK activity is involved in osteoblast function *in vitro* and regulation of bone mass *in vivo*. This would position AMPK as a novel molecular coordinator of bone and energy metabolisms and as an attractive therapeutic target for osteoporosis.

Material and methods

Chemicals

Ghrelin was obtained from NeoMPS (Strasbourg, France) and compound C was bought from Calbiochem (Nottingham, UK). Phospho (Thr 172)-AMPK and total AMPK antibodies detecting both the α -1 and α -2 catalytic subunits were purchased from New England Biolabs (Hitchin, UK). β -actin (1-19) antibody was purchased from Dako (Ely, UK). Propidium iodide staining solution (PI stain) was purchased from INCYTO (Seoul, South Korea). All other hormones and chemicals were purchased from Sigma Aldrich (Dorset, UK).

Ros 17/2.8 cell culture and treatment with AMPK modulators

The rat osteosarcoma cell line ROS 17/2.8 was cultured in Dulbecco's Modified Essential Medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin at 37 °C and 5% CO₂. The medium was changed twice a week and ROS 17/2.8 cells were used for experiments after reaching 80–90% confluence. For AMPK modulators and hormone treatments, cells were either plated in 6-well culture plates (NUNC, Dossel, Germany) at a concentration of 25,000 cells/ml for Western Blotting and alkaline phosphatase activity, or at a concentration of 50,000 cells/ml in 60 mm dishes (NUNC) for AMPK activity. Culture medium was replaced by medium containing 2% FCS 24 h before treatment.

Ros 17/2.8 cell proliferation

Cell proliferation was measured by staining with the nuclear PI stain and total number of cells counted using an automated "In Cyto" cell counting system. Briefly, ROS 17/2.8 cells (25,000 cells/ml) were cultured on custom-made tissue culture-treated plastic slides (NUNC) placed in 4-well plates purchased from quadriPERM, (Greiner bio-one, Stonehouse, UK). They were cultured in 2% FCS for 24 h prior to being treated with AMPK modulators for 24 h. Cells were rinsed with sterile PBS and fixed in ice cold methanol (VWR International, Lutterworth, UK) for 10 min. Staining solution (PI stain solution in a ratio of 1:3 with dH₂O) was then added to each slide and incubated at room temperature for 10 min. The excess dye was removed, dH₂O added and the slides left in the dark prior to counting. Total

cell number was determined by the measurement of the amount of DNA staining by fluorescence using a microchip type automatic cell counter machine according to manufacturer's instructions (INCYTO).

Alkaline phosphatase activity (ALP) assay and staining

ROS 17/2.8 cells in 6-well plates were treated for 48 h with AMPK modulators. Cells were rinsed three times with PBS, digested with 0.1% triton X-100, scraped off the plate and the cell suspension sonicated (Ultrasonic disintegrator, MSE laboratories, Butte, MT). The protein assay was performed with the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). ALP was assayed by a method modified from that of Lowry et al. [47]. Enzyme activity assay was performed in assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$ and 2 mM p-nitrophenylphosphate pH 10.5) added to cell homogenates. A standard curve was prepared with p-nitrophenol solutions. Incubation of standards and samples was performed at 37 °C for 5 min and the reaction stopped by adding 0.3 M NaOH. Reaction mixtures were transferred into 96-well plates and absorbance measured at 410 nm using a plate reader. Relative ALP activity is defined as millimoles of p-nitrophenol phosphate hydrolyzed per minute per milligram of total protein. ALP activity was also determined by histochemical staining. Briefly, ROS17/2.8 cells were fixed in 4% paraformaldehyde, rinsed and treated with substrate naphthol AS-TR which is subsequently hydrolysed in the presence of the enzyme. The introduction of fast red dye produces a coloured precipitate.

RT-PCR

Right and left mouse tibiae were carefully dissected and all their surrounding musculature removed leaving the periosteum intact. The cartilaginous ends of the bones were removed and the remaining tibial shaft spun at 5000 rpm for 2 min to remove the marrow. The tibial shafts were then snap-frozen in liquid nitrogen, pulverized under liquid nitrogen using a mortar and pestle and lysed in Qiazol lysis reagent (Qiagen Ltd., Crawley, UK). Rat cranial tibial muscles were similarly snap-frozen in liquid nitrogen and lysed in Qiazol lysis reagent. Total RNA was extracted from these lysed samples and from ROS 17/2.8 cells using RNeasy mini kit (QIAGEN, Crawley, UK). RNA integrity was verified by electrophoresis using ethidium bromide staining and by OD_{260}/OD_{280} nm absorption ratio (>1.95). Total RNA (4 μ g) was reverse transcribed with 200 U of SuperScript II RNase reverse transcriptase (Invitrogen), using 50 ng random primers (Invitrogen, Paisley, UK). PCR was carried out according to PCR kit (Invitrogen) protocol using approximately 0.6 μ g cDNA, 2 U taq polymerase and 0.2 μ M forward and reverse primer mix. Forty cycles of denaturation were performed with an annealing temperature of 56 °C. PCR products were verified by electrophoresis using ethidium bromide staining. Primer sequences are summarized in Table 1.

Protein extraction and Western Blotting

ROS 17/2.8 cells were briefly washed in ice cold PBS and lysed in denaturing lysis buffer (2% SDS, 2 M urea, 8% sucrose, 20 mM sodium glycerophosphate, 1 mM NaF, and 5 mM Na_2VO_4). Proteins were denatured by boiling for 5 min and concentrations determined by BCA protein assay. 10 μ g of proteins was size-fractionated using SDS-PAGE and electrotransferred onto Protran nitrocellulose membranes (Schliecher and Schuell, Dassel, Germany). Membranes were blocked for 1 h in 0.2% (w/v) I-block (Topix, Bedford, MA), before being incubated with antibodies. The blots were incubated overnight at 4 °C with antibodies against total AMPK, phospho (Thr 172)-AMPK, β -actin at a 1:1000 dilution. Proteins were visualised using the enhanced chemiluminescence detection system (ECL) (GE Healthcare UK Ltd, Little Chalfont, UK). The intensity of the specific bands was quantified by densitometry using Image J software.

Kinase assay for AMPK

Immunoprecipitate kinase assay for AMPK was determined by phosphorylation of SAMS, a synthetic peptide substrate of AMPK, as described previously [25]. Briefly, cells were lysed in AMPK lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X100, 1 mM DTT, 1 mM benzamide, 1 mM phenylmethane sulfonyl fluoride (PMSF), 5 µg/ml soybean trypsin inhibitor) and the protein content determined by BCA protein assay. 200 µg of protein was immunoprecipitated using protein G beads (Amersham Biosciences, Bucks, UK) and a mixture of $\alpha 1$ and $\alpha 2$ AMPK antibodies (2.5 µg/sample of each). The immunoprecipitate was divided into three aliquots. Two were assayed for AMPK activity with a reaction solution containing 0.1 µCi of [γ - 32 P] ATP, 0.1 µl of 100 mM cold ATP, 0.25 µl of 1 M MgCl₂, 10 µl of 1 mM AMP and 10 µl of 0.1 mM SAMS (Upstate Biotechnology, Dundee, Scotland), while the third aliquot was assayed with the same mixture except that SAMS was replaced by buffer. The reaction was performed on a shaker for 20 min at 30 °C and samples were pipetted onto paper squares (P81, Upstate Biotechnology). The reaction was stopped by placing the paper squares into 1% phosphoric acid and after repeated rinsing the activity was counted using a scintillation counter. AMPK activity was calculated as nanomoles of phosphate transferred to the SAMS peptide per minute and per mg of protein.

Primary rat calvaria osteoblast culture and in vitro bone formation assay

Primary rat osteoblasts were obtained by sequential enzyme digestion of calvarial bones from 2-day-old Sprague-Dawley rats, as previously described [48]. The first two digests were discarded and the cells were resuspended in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin. Cells were cultured for 2–4 days at 37 °C, 5% CO₂ in 75 cm² flasks until they reached confluence. Upon confluence, rat primary cells were sub-cultured into 6-well plates in DMEM supplemented with 2 mM β -glycerolphosphate, 50 mg/ml ascorbic acid and 10 nM dexamethasone with half medium changes every 3 days. Cells were treated with AMPK stimulators (AICAR and metformin) at concentrations ranging from 0.5 µM to 100 µM or with AMPK inhibitor compound C (concentrations from 0.05 to 1 µM), which were added to the medium at each change. In some experiments cells were pre-treated with compound C for 30 min before exposure to AICAR. Cells were cultured for up to 21 days. Bone nodule formation was measured as previously described [48]. Briefly, cell layers were fixed in 2% glutaraldehyde for 5 min. Mineralised bone nodules were visualised by staining with alizarin red (1% solution in water) for 5 min. The plates were imaged at 750 dpi, using a high-resolution flat-bed scanner (Epson Photo 4200). Binary images of each individual well were then subjected to automated analysis (Image J, <http://rsbweb.nih.gov/ij/>) using constant 'threshold' and 'minimum particle' levels, to determine the number and plan surface area of mineralised bone nodules as described previously [48]. Some cultures were stopped at 7 and 14 days to assess alkaline phosphatase activity, as described above.

Micro-CT analysis of tibia

AMPK $\alpha 1$ –/– and AMPK $\alpha 2$ –/– mice were generously provided by Benoit Violet (INSERM U567, Paris) and were generated as previously described [49,50]. Tibia were harvested from 4 month-old male wild type (WT) and AMPK $\alpha 1$ –/– KO mice ($n=10$ /group) and from 3 month-old male WT and AMPK $\alpha 2$ –/– mice ($n=6$ /group). Tibiae were scanned with high-resolution (5 µm pixel size) micro-computed tomography (micro-CT, Skyscan 1172, Belgium), as previously described [51]. The whole tibiae were reconstructed using NRecon v.1.4.4.0 (Skyscan, Belgium) and bone histomorphometric analyses in 2- and 3-dimensions (2D, 3D) were performed by Skyscan software (CT-Analyser v.1.5.1.3). For the analysis of trabecular bone, the cortical shell was excluded by operator-drawn regions of interest and 3D algorithms were used to determine the relevant parameters which included: Bone

Volume Percentage (BV/TV), Trabecular Thickness (Tb.Th), Trabecular Number (Tb.N), Trabecular Spacing (T.Sp), Structure Model Index (SMI), Trabecular Pattern Factor (TPf) and Degree of Anisotropy (DA). Analysis of cortical bone was performed using a 0.49 mm long segment (or 100 tomograms) at 37 percent of the tibias' length from its proximal end. For analysis of the cortical bone compartment, 2D computation was used and parameters were determined for each one of the 100 tomograms and then averaged. They included: Tissue Area (T.Ar), Bone Area (B.Ar), Medullary Area (M. Ar), Periosteal Perimeter (P.Pm), Endosteal Perimeter (E.Pm), Cortical Thickness (Ct.Th), Mean Polar Moment of Inertia (MMIp) and Eccentricity (ECC). Coefficients of variation (CVs) were determined by re-analysing the trabecular bone on the same sample five times on separate occasions to account for operator bias and determine reproducibility of the analysis. Only the trabecular bone analysis was chosen, due to the greater margin of error involved in drawing the regions of interest, since the cortical shell shape changes markedly through the region analysed. The CV of each parameter was determined as the ratio between the standard deviation and the mean. The CVs for relevant parameters are the following: BV/TV: 1.65%; Tb.Th: 0.85%; Tb. Sp: 0.85%, and Tb.N: 1.72%.

Statistics

Statistically significant differences were determined using One Way ANOVA test or Mann Whitney *U* test (for KO analyses) with SPSS software, using $p < 0.05$ as significant.

Results

Expression of AMPK subunits in ROS 17/2.8 osteoblastic cells and mouse tibia

We performed RT-PCR analysis using primers directed against murine sequences to examine mRNA expression of AMPK subunits in ROS17/2.8 osteoblastic cells and mouse tibia. We showed that bone cells and tissue contain isoforms $\alpha 1$, $\beta 1$ and $\beta 2$, $\gamma 1$, $\gamma 2$ and $\gamma 3$ (Fig. 1), suggesting possible combinations of isoforms to form different AMPK complexes. The $\alpha 2$ subunit was not detected in tibia and ROS17/2.8 cells, indicating that $\alpha 1$ complexes are dominant in bone. In contrast, all AMPK subunits were expressed in rat skeletal muscle, including $\alpha 2$ (Fig. 1).

Regulation of AMPK activity in ROS 17/2.8 osteoblastic cells

To investigate whether AMPK is activated in ROS17/2.8 osteoblastic cells in response to known modulators of AMPK activity, cells were exposed for 1 h to two known activators of AMPK, the cell-permeable AMP analogue AICAR and metformin, as well as to the AMPK inhibitor compound C. Figs. 2A and B show that AICAR and metformin dose-dependently stimulate AMPK α phosphorylation at the Thr 172 while total AMPK levels did not change. In contrast, compound C decreases AMPK phosphorylation (Fig. 2C). Quantification of the bands on the blots showed that the increases in phosphorylation were significant. In agreement with these changes in AMPK phosphorylation, using a functional AMPK assay, we showed that AICAR and metformin enhanced AMPK activity in ROS 17/2.8 osteoblastic cells (Figs. 2D and E), confirming AMPK activation in osteoblastic cells by known AMPK activators. We then tested if AMPK phosphorylation and activity are modulated in osteoblastic cells by hormones and drugs that affect their function. We tested the effect of ghrelin, a gastric peptide hormone known to regulate AMPK activity in brain and several peripheral tissues and to stimulate body weight as well as osteoblast differentiation and function [7,36]. Fig. 3 illustrates that ghrelin increases phosphorylation of AMPK (Fig. 3A) and stimulates AMPK activity (Fig. 3C) in ROS17/2.8 cells. Given the importance of the SNS in controlling bone formation [1,2] and the previous demonstration that β -adrenergic stimulation can modulate AMPK in adipocytes [7], we examined the effect of propranolol, a beta-adrenergic receptor antagonist that stimulates bone formation [2], on AMPK activity in

ROS 17/2.8 cells. Our results demonstrate that propranolol enhances AMPK phosphorylation and activity in those cells (Figs. 3 B and D).

Effect of AMPK activation on ROS 17/2.8 cell proliferation and differentiation

To investigate the effects of AMPK stimulators on ROS 17/2.8 cell proliferation, cells were treated with various concentrations of AICAR and metformin for 24 h following overnight serum starvation, and then incubated with the nuclear stain propidium iodide as described in Material and methods section. Metformin treatment did not induce any change in osteoblastic cell proliferation (Fig. 4B), while AICAR significantly inhibited ROS 17/2.8 cell proliferation but only at a high concentration (Fig. 4A). To examine the effect of AMPK stimulation on osteoblastic cell differentiation, ALP was evaluated. We used two methods, measurement of the phosphatase activity in the cell lysates using p-nitrophenyl-phosphate as a substrate and staining of alkaline phosphatase with Fast-red TR. ALP was dose-dependently inhibited by AICAR (Figs. 5A,B). Lower dose of AICAR (50 μ M) had no effect on ALP activity (not shown). In contrast, metformin had no effect on alkaline phosphatase activity in ROS 17/2.8 cells assayed by these two methods (Figs. 5C,D).

Effect of AMPK activation on in vitro bone formation

The effect of AMPK modulators was tested on bone nodule formation by cultured rat osteoblasts. In this model, abundant mineralised bone nodules with characteristic trabecular features form after 2 to 3 weeks of culture [48, Fig. 6A]. Both AICAR and metformin dose-dependently stimulate bone nodule formation (Figs. 6B,C), while compound C dose-dependently inhibits bone formation (Fig. 6D). The effects were visible in alizarin red-stained cultures and the quantitative analysis confirmed the potent dose-dependent stimulatory effects of AMPK activators and the inhibition induced by compound C on the number and size of mineralised bone nodules formed by osteoblasts (Fig. 6A). When primary osteoblasts were co-treated with AICAR and compound C, compound C suppressed the stimulatory effect of AICAR on bone nodule formation (Fig. 6E). Interestingly, alkaline phosphatase activity was increased by AICAR treatment during the mineralising phase of culture (Fig. 6F).

Analysis of the bone phenotype of AMPK α 1 $^{-/-}$ and AMPK α 2 $^{-/-}$ mice

To determine the bone phenotype of mice lacking AMPK α 1 and AMPK α 2, micro-CT scanning analysis of tibiae was performed. Both cortical and trabecular bone compartments were smaller in the AMPK α 1-deficient mice compared to the WT mice (Fig. 7). The α 1-knockout mice showed dramatic decreases in trabecular bone volume (BV/TV) by 31.1% (A), trabecular number (Tb.N) by 26% (B), and trabecular thickness (Tb.Th) by 7% (C). Trabecular separation (T.Sp) was significantly increased, as well as trabecular pattern factor (TPf) and structure model index (SMI) which are two parameters reflecting respectively the trabecular interconnection and trabecular shape, plate to rod elements (not shown). There was no significant difference in the degree of anisotropy (DA) between WT and KO (not shown). The cortical indexes were also decreased in mice lacking AMPK α 1. B. Ar (Fig. 7D) and Ct.Th (Fig. 7F) were significantly decreased in mice lacking AMPK α 1 but medullary area was not affected (Fig. 7E). P.Pm and MMIp were also significantly decreased in the KO mice while the eccentricity (Ecc) of the cortex in respect to its cross-sectional centre of gravity remained unchanged (not shown). There was no significant difference between tibia lengths of AMPK α 1-deficient mice and WT mice (data not shown). We also analysed the tibia of AMPK α 2 KO mice by micro-CT. AMPK α 2 subunit-knockout mice had no significant changes in cortical and trabecular bone parameters compared with WT mice (not shown).

Discussion

The involvement of AMP kinase (AMPK) signalling in osteoblastic and adipocytic differentiation and function has recently attracted considerable interest due to the convergence between bone and fat metabolism [3,52]. Our study is the first one to examine the interaction between AMPK and ghrelin in osteoblasts and the effect of AMPK activation on bone formation by primary osteoblasts *in vitro*. The majority of AMPK functions in cells have been described on the basis of cell incubation with pharmacological activators of AMPK such as 5-aminoimidazole-4-carboxamide riboside (AICAR) and the anti-diabetic drug metformin [53]. AICAR directly activates AMPK by generating intracellularly ZMP, a nucleotide with close similarity to AMP, while metformin indirectly activates AMPK by increasing the intracellular AMP/ATP ratio and subsequently making AMPK a better substrate for LKB1, an AMPK upstream kinase [54]. Both AICAR and metformin enhanced AMPK phosphorylation and activity in ROS 17/2.8 cells following 1 h treatment, indicating that they can stimulate the AMPK signalling pathway in those cells. As previously reported [29], high concentrations of metformin and AICAR are required to give a large activation of AMPK in short-time experiments. Our preliminary results also indicate sustained AMPK phosphorylation in response to AICAR and metformin after 24 h treatment. Although the AMPK signalling pathway has not been examined in ROS17/2.8 osteoblastic cells, it was previously studied in MC3T3-E1 cells and shown to involve an inhibition of HMG-CoA reductase, an enzyme of the mevalonate pathway engaged in bone formation, eNOS and BMP-2 gene expression [28].

AMPK has a broader role in metabolism through the control of appetite both at the cellular and whole body levels [7,8,23,24] and many appetite-regulating hormones activate AMPK. The regulation of AMPK activity in bone cells by hormones that affect bone and energy metabolism has not been studied. However, one recent study shows that adiponectin, which is highly expressed in adipose tissue, can phosphorylate AMPK and stimulate the differentiation and mineralisation of MC3T3-E1 cells [27]. Among the hormones that control appetite, ghrelin is a potent appetite stimulator which also affects bone mass, indirectly by stimulating growth hormone (GH) secretion both *in vivo* and *in vitro* and directly by affecting osteoblast proliferation and differentiation [36,55]. In addition, the effects of ghrelin on food intake and energy homeostasis are linked to AMPK activity [7,23,56]. We have shown that ghrelin can stimulate AMPK phosphorylation and activity in ROS17/2.8 cells, suggesting that the AMPK signalling pathway may be involved in the regulation of osteoblast function by ghrelin. Another metabolic modulator of AMPK in osteoblasts is the beta-adrenergic component of the SNS. Our study clearly demonstrates that the beta-blocker propranolol, known to stimulate bone formation both *in vivo* and *in vitro* by suppressing β 2-adrenoreceptor signalling in osteoblasts [2], also stimulates AMPK phosphorylation and activity in ROS 17/2.8 cells. AMPK activation is known to mediate the effects of β 2-adrenoreceptor stimulation in adipocytes as well as many peripheral metabolic and cardiac effects of ghrelin [7,25,57], indicating that AMPK signalling may be an essential mediator of the metabolic effects of hormones and neuromediators that affect both bone and fat metabolism.

To date, only a few studies have investigated the role AMPK activation in osteoblast function, and most of them have used MC3T3-E1 mouse calvaria-derived cells. For the first time, we have chosen to use the osteoblastic cell line ROS 17/2.8 which expresses many of the osteoblastic features to investigate the effect of AMPK activation on osteoblast proliferation and differentiation, and primary osteoblasts derived from rat calvaria to study the effect of AMPK activation on *in vitro* bone formation. AMPK activation has been shown to suppress cell proliferation in both malignant and non-malignant cells via cell cycle regulation and inhibition of protein and fatty acid synthesis [58]. We have shown that

treatment with metformin does not affect ROS 17/2.8 cell proliferation, while AICAR inhibits cell proliferation but only at a high concentration of 500 μ M. As cell apoptosis was not investigated, we can't exclude that this high concentration of AICAR induces some apoptosis in ROS 17/2.8 cells. AICAR has indeed been shown to be a very potent inhibitor of tumoral cell proliferation, inhibiting the phosphorylation of AKT and mTOR, which are important kinases regulating cell growth and survival [59]. While metformin can also activate the AMPK pathway to induce tumoral cell cycle arrest, other pathways may be required [60]. Our findings are in contradiction with the data from Cortizo et al. [41] which demonstrate that metformin stimulates proliferation of UMR-106 and MC3T3-E1 osteoblastic cell lines at similar concentrations. In addition, Kanazawa et al. [27] showed that AICAR stimulates proliferation of MC3T3-E1 cells. In agreement with these previous results, Zhen et al. [61] demonstrated that metformin, at a similar concentration range, markedly increases proliferation of rat primary osteoblasts using the MTT assay. However, similarly to our results, metformin had no effect on cell proliferation in many other cell types including smooth muscle cells and endothelial cells [62,63]. This inconsistency regarding the proliferative effect of metformin and AICAR in different cell types as well as among various osteoblastic cell lines is still unclear and requires further investigation. Most studies have investigated the effect of AMPK agonists in MC3T3-E1 osteoblastic cells that are non-transformed cells. In contrast, ROS 17/2.8 cells are malignant cells and may respond differently with regards to growth rate. As the proliferative capacity of cells depends on glucose concentrations and then glucose transport [61,64], it may also be possible that AMPK activation in different cell types is affected by variations in their sensitivity to glucose. This divergence between studies was also observed for the effect of AMPK activation on osteoblast differentiation. While several studies showed that AMPK activation stimulates differentiation and mineralisation of osteoblastic MC3T3-E1 cells [27,28,41], recent work demonstrated that osteoblast differentiation in both primary osteoblasts and MC3T3-E1 cells was associated with decreased AMPK activity [29]. Our results demonstrate that ALP in ROS 17/2.8 cells, which is used to evaluate osteoblastic differentiation, is decreased by AICAR while not affected by metformin treatment. This suggests that although AICAR and metformin both stimulate AMPK activation in ROS17/2.8 cells, they also show different mechanisms of action on ROS 17/2.8 cell differentiation. Zhou et al. [65] have shown that membrane permeability of metformin is a time-dependent and slow process and that to have the same AMPK stimulation than AICAR, higher concentrations of metformin and longer treatment are required. Using identical concentrations of metformin than in our study, Cortizo et al. [41] have not shown any effect of metformin on alkaline phosphatase activity in UMR106 osteoblastic cells. No effect of metformin on osteoblast differentiation at any concentration was also reported by Kasai et al. [29]. In contrast to the quantification of ALP after 15 days of osteogenic differentiation in MC3T3-E1 cells and primary osteoblasts, we measured ALP in ROS 17/2.8 cells following 48 h treatment with AICAR or metformin. To clarify those discrepancies and examine the long-term effect of AICAR and metformin on osteoblast differentiation and activity, we used an *in vitro* model of bone formation in which the ultimate function of osteoblasts, namely the production and the mineralisation of a bone matrix, can be assessed quantitatively [48]. In this model both treatments with AICAR and metformin for 3 weeks dose-dependently stimulated bone nodule formation, while AMPK inhibitor compound C dose-dependently reduced bone formation. Those effects were observed at doses of AICAR and metformin ranging between 0.5 100 μ M. Doses higher than this range (such as 200 and 500 μ M used for ROS17/2.8 cell proliferation and differentiation) had a toxic effect in this model. Interestingly, we found that AICAR had no effect on alkaline phosphatase activity after 7 days of culture, while it stimulated alkaline phosphatase activity after 14 days, during the mineralising phase of culture. This is in agreement with the stimulatory effect of AICAR on bone nodule formation but in contrast to the inhibitory effect of AICAR on alkaline phosphatase activity in ROS17/2.8 cells. This divergence could be due to the long-term

AICAR treatment, to different cellular uptakes and mechanisms of action of AICAR in primary compared to tumoral osteoblasts or, alternatively, to the stage of osteoblast differentiation. Our results suggest that sustained AMPK activation could be beneficial for bone formation. The demonstration that compound C suppresses the effect of AICAR on bone nodule formation supports this hypothesis. These findings are in line with the demonstration that both ghrelin and propranolol stimulate AMPK activation and enhance bone formation [2,36]. However, the signalling pathways linking AMPK activation and bone formation have not yet been established and therefore the physiological meaning of AMPK activation in osteoblasts is still unknown. Changes in metabolic pathways occur during bone cell differentiation to allow high ATP generation for bone matrix production and mineralisation, and AMPK may be the signal to sense osteoblast energy status [66]. AMPK activation may also be involved in the relationship between fat and bone in the marrow, which may vary according to energy needs and shift from osteoblastogenesis to adipogenesis depending on the environmental conditions. In support of this hypothesis, AMPK was suggested to be involved in the inhibition of adipogenesis [67].

The three subunits of AMPK have different functions. The α subunits contain the conventional kinase domain at their N-terminus while the C-terminal regions are required to form a complex with the β and γ subunits. The β and γ regulatory subunits help to maintain the stability of the AMPK complex but are also known to have other functions, including binding of glycogen and regulatory nucleotides AMP and ATP [15]. We demonstrated that except $\alpha 2$, all isoforms of AMPK are expressed in osteoblastic cells and in tibia, implying that multiple forms of AMPK complexes exist in skeletal tissue. In contrast with skeletal muscle, heart and liver, and similar to adipose tissue, $\alpha 1$ is the dominant isoform expressed in bone, suggesting that this isoform may play a major function in skeletal metabolism. This is confirmed by the low bone mass phenotype observed in the tibiae of mice deficient for $\alpha 1$ subunit. Both cortical and trabecular bone parameters were lower in AMPK $\alpha 1$ -deficient mice compared with WT mice, suggesting that the knockout mice have impaired bone metabolism. It is unlikely that these skeletal defects in these knockout mice are a consequence of previous developmental or growth alterations as there is no difference in bone length between AMPK $\alpha 1$ -deficient mice and WT mice. No difference in body mass was reported either, but AMPK $\alpha 1$ -deficient mice have less fat [68]. However, these mice respond normally to high fat diet [69], and energy expenditure and food intake are not modified in AMPK $\alpha 1$ -deficient mice [70]. No known metabolic changes were detected in AMPK $\alpha 1$ -deficient mice that could explain our skeletal defects [50,71]. Our results rather suggest that AMPK $\alpha 1$ isoform is important for bone mass maintenance. In agreement with the absence of AMPK $\alpha 2$ in bone, our preliminary data showed no cortical and trabecular bone abnormalities in AMPK $\alpha 2$ -deficient mice, suggesting that the AMPK $\alpha 2$ -isoform is not critical for bone metabolism. As cellular functions have not yet been examined in the AMPK $\alpha 1$ -knockout mice, we did not elucidate whether bone abnormalities are mainly due to bone resorption or bone formation defects. Our *in vitro* results suggest, however, that AMPK activity is important for bone formation. While our data are the first to show a decrease in bone mass in AMPK $\alpha 1$ knockout mice, recent published data show that germline deletion of AMPK regulatory β -subunits also resulted in reduced trabecular bone density and bone mass [72]. Altogether, our data confirm that AMPK activity has an important role to play in the regulation of bone metabolism.

We have shown that AMPK activity can be regulated in bone cells by known modulators of AMPK and by hormones that control bone mass, confirming the existence of a hormonal link between bone mass and energy metabolism. We have also demonstrated that AMPK activity is important for bone nodule formation *in vitro* and the maintenance of bone mass *in vivo*, further supporting a role for AMPK signalling in skeletal physiology.

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Fig. 1.

AMPK subunit mRNA expression in ROS 17/2.8 osteoblastic cells and tibiae. Right and left mouse tibiae were carefully dissected. The cartilaginous ends of the bones were removed as well as the marrow. The tibial shafts were then snap-frozen in liquid nitrogen. Rat cranial tibial muscle was dissected and similarly snap-frozen in liquid nitrogen. ROS17/2.8 cells were grown in DMEM containing 10% FCS until sub-confluent. Total RNA was extracted from whole tissues and cell lysates and subjected to RT-PCR analysis using specific primers of AMPK subunits.

Fig. 2.

Effect of AICAR, metformin and compound C on AMPK activation in ROS 17/2.8 osteoblastic cells. Semi-confluent ROS17/2.8 cells were incubated in medium containing 2% FCS overnight and then treated for 1 h with various concentrations of AICAR (100–500 μ M), metformin (100–500 μ M) and compound C (5–20 μ M). Whole cell lysates were collected for Western Blotting or immunoprecipitation. A–C. ROS 17/2.8 cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies directed against AMPK α phosphorylated at Thr 172 and AMPK α . Representative immunoblots are shown, repeated with similar results twice (AICAR and compound C) or 3 times (metformin). All blots were quantified using image J and the p-AMPK:total AMPK ratio was determined for each experiment. Results shown are representative of two or three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. D–E. Total AMPK was immunoprecipitated from lysates and assayed for AMPK activity determined by phosphorylation of SAMs. Results shown (mean $\pm$ SEM, $n=6$) are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 3.

Neuroendocrine activation of AMPK in ROS 17/2.8 cells. Semi-confluent ROS17/2.8 cells were incubated in medium containing 2% FCS overnight and then treated for 1 h with various concentrations of ghrelin (10^{-9} – 10^{-7} M) and propranolol (10^{-8} – 10^{-6} M). Whole cell lysates were collected for Western Blotting, resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies directed against AMPK α phosphorylated at Thr 172 and AMPK α . Representative immunoblots for ghrelin (A) and propranolol (B) are shown, repeated twice with similar results. All blots were quantified using image J and the p-AMPK:total AMPK ratio was determined for each experiment. Results shown are representative of two independent experiments. * p <0.05; ** p <0.01; *** p <0.001. C–D. Total AMPK was immunoprecipitated from lysates and assayed for AMPK activity determined by phosphorylation of SAMS. Results shown (mean $\pm$ SEM, n =6) are representative of two independent experiments. * p <0.05; ** p <0.01, when compared with untreated control.

Fig. 4.

Effect of AMPK activators on ROS 17/2.8 cell proliferation. ROS 17/2.8 cells cultured on custom-made plastic slides were incubated in medium containing 2% FCS for 24 h prior to being treated with different concentrations of AICAR (A) and metformin (B) for 24 h. 20% FCS was used as a positive control. Cell proliferation was evaluated by cell staining with the nuclear stain propidium iodide and by counting the total number of cells using an automated “In Cyto” cell counting system. Data shown (mean $\pm$ SEM, $n=6$) are representative of two independent experiments. ** $p<0.01$; *** $p<0.001$, when compared with untreated control.

Fig. 5.

Effect of AMPK activators on alkaline phosphatase activity in ROS 17/2.8 cells. ROS 17/2.8 cells were incubated in medium containing 2% FCS overnight prior to being treated with different concentrations of AICAR (A) and metformin (C) for 48 h. Dexamethasone (10^{-8} M) was used as a positive control. ALP was measured using p-nitro-phenyl phosphate as a substrate and normalised to total protein content or visualised by staining as described in Material and methods section. Data shown (mean $\pm$ SEM, $n=6$) are representative of two independent experiments. *** p 0.001 when compared to control. Below the graphs are representative figures of three independent experiments illustrating ALP staining with identical concentrations of AICAR (B) and metformin (C).

Fig. 6. Effect of AMPK modulators on *in vitro* bone formation. Primary osteoblasts obtained from rat calvaria by trypsin/collagenase digestion were cultured for 14–17 days in the presence of different concentrations of AICAR, metformin and compound C. In some experiments cells were pre-treated with compound C for 30 min before exposure to AICAR. Formation of ‘trabecular-shaped’ bone nodules was evaluated following alizarin red staining and quantified by densitometric analysis using IMAGE J software. (A) Representative low-power images of alizarin red-stained nodules formed by rat calvarial osteoblasts cultured for 17 days in the presence of AICAR, metformin and compound C. The appearance of cultures at higher magnification is shown. (B,C) Stimulatory effects of AICAR (B) and metformin

(C) on bone nodule formation. Data shown (mean $\pm$ SEM, $n=6$) are representative of six independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, when compared with untreated control. (D) Inhibitory effect of compound C on bone nodule formation. Data shown (mean $\pm$ SEM, $n=6$) are representative of two independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, when compared with untreated control. (E) Inhibition by compound C of the stimulatory effect of AICAR on bone nodule formation. Data shown (mean $\pm$ SEM, $n=6$) are representative of two independent experiments. ** $p<0.01$; *** $p<0.001$, when compared with AICAR. ### $p<0.001$, when compared with untreated control. (F) Primary osteoblasts were incubated with different concentrations of AICAR for 7 or 14 days. ALP was measured using *p*-nitro-phenyl phosphate as a substrate and normalised to total protein content. Data shown (mean $\pm$ SEM, $n=6$) are representative of two independent experiments. * $p<0.05$; *** $p<0.001$, when compared to control.

Fig. 7.

Bone phenotype of AMPK α 1 subunit-knockout mice. Trabecular and cortical microarchitecture assessed by micro-CT in WT and AMPK α 1 subunit-knockout mice aged 4 months. (A,B,C) 3-dimensionally computed BV/TV (A), Tb.N (B) and Tb.Th (C) in the proximal metaphysis of WT and AMPK- α 1 knockout mice. (D,E,F) 2-dimensionally computed bone area (D), medullary area (E) and Ct.Th (F) in the mid-diaphysis cortical bone of WT and AMPK- α 1 knockout mice. Bars represent mean $\pm$ SEM of $n=10$ mice/group. * $p<0.05$,

Table 1

Reverse transcriptase PCR primer sequences (5'→3') for AMPK subunits.

NCBI reference sequence ID	Gene	Primer sequence 5'-3'
NM 019142.1	PRKAA1 forward	CTCTATGCTTTGCTGTGTGG
	PRKAA1 reverse	GGTCCTGGTGGTTTCTGTG
NM 023991.1	PRKAA2 forward	TCGCAGTGGCTTATCATCTC
	PRKAA2 reverse	TGTCGTATGGTTTGCTCTGG
NM 031976.1	PRKAB1 forward	TCAAGGATGGAGTGATGGTG
	PRKAB1 reverse	GACTATGTGGGGTGAGATG
NM 022627.1	PRKAB2 forward	AAACTCACTGGGCGAGGAAC
	PRKAB2 reverse	CCACACAGCCAATACACAGG
NM 013010.2	PRKAG1 forward	GCTACAGATTGGCACCTACG
	PRKAG1 reverse	TCAGGGCTTCTTCTCTCCAC
NM 184051.1	PRKAG2 forward	GCCTTCTTTGCTTTGGTAGC
	PRKAG2 reverse	GCTCATCCAGGTTCTGCTTC
NM 001106921.1	PRKAG3 forward	TCACCATCACGGACTTCATC
	PRKAG3 reverse	CATCAAAGCGGGAGTAGAGG